UNIVERSITY OF ALBERTA

THE MODULATION OF L-TYPE CALCIUM CHANNEL CURRENTS BY PTHrP IN OSTEOBLAST-LIKE CELLS

by

MEGAN LALONDE

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in partial fulfilment of the requirements for the degree of

Master of Science

DEPARTMENT OF PHYSIOLOGY

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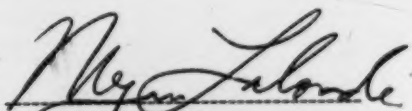

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ABSTRACT

Parathyroid hormone (PTH) affects osteoblasts and stimulates bone resorption. Parathyroid hormone-related protein (PTHrP), which is responsible for the humoral hypercalcemia of malignancy (HHM), is also important in bone metabolism. The concentration of PTHrP in blood is lower than that of PTH; nevertheless, PTHrP is widely distributed in tissues and has paracrine, autocrine, and endocrine mechanisms of action. In this thesis, the effects of PTHrP and PTH on L-type Ca^{2+} channel currents and Ca^{2+} channels in UMR 106 cells were investigated using the patch clamp technique. PTHrP increased L-type Ca^{2+} channel currents and the increase was concentration-dependent. PTHrP produced a maximal increase of 122.3±27% at a concentration of 6.25×10^{-8}M. PTH had a less potent effect on the L-type Ca^{2+} current, as it produced a maximal increase of 99.2±24% at a concentration of 7.5×10^{-6}M. Single channel measurements, using the cell-attached version of the patch clamp technique showed an increase in both the number of channel openings and the open time of the channels in the presence of PTHrP. This suggests that PTHrP affects the gating of L-type Ca^{2+} channels in UMR 106 cells. The PTHrP-induced increase in the L-type Ca^{2+} channel current was not positively modulated by cyclic AMP/protein kinase A, protein kinase C, or cyclic GMP/protein kinase G, since activation of all of these kinases decreased the L-type current. In addition, PTHrP and PTH increased intracellular cAMP ([cAMP]), as a function of concentration, suggesting that PTHrP induces its bone resorption effect through multiple signal pathways.

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TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction 1

1.2 The discovery and history of PTH and PTHrP 1

1.3 The structure of PTH and PTHrP 4

1.4 Synthesis and secretion of PTH and PTHrP 5

1.5 Physiological actions of PTH and PTHrP 9

1.5.1 Cellular actions of PTH 11

1.5.1.1 Cellular actions of PTH on bone 11

1.5.1.2 Cellular actions of PTH on the kidney 13

1.5.1.3 Cellular actions of PTH on the cardiovascular system 13

1.5.2 Cellular actions of PTHrP 14

1.5.2.1 Cellular actions of PTHrP on bone 14

1.5.2.2 Cellular actions of PTHrP on the kidney 16

1.5.2.3 The role of PTHrP in fetal development 16

1.5.2.4 The role of PTHrP in lactation 17

1.5.2.5 The role of PTHrP as a cytokine 17

1.5.2.6 The role of PTHrP in the cardiovascular system 18

1.6 Actions of PTHrP and PTH on intracellular signalling systems 19

1.6.1 PTH and PTHrP receptors 22

1.6.2 Effects of PTHrP and PTH on intracellular calcium concentration 23

1.6.3 Effects of PTHrP and PTH on the adenylate cyclase/protein kinase A (PKA) signal transduction pathway 27

1.6.4 Effects of PTHrP and PTH on the phospholipase (PLC)/protein kinase C (PKC) signal transduction pathway 30

1.6.5 The interaction of the intracellular signalling pathways 33

1.7 Voltage-dependent calcium channels 34

1.7.1 L-type calcium channels in bone 35

1.7.2 Significance of studying L-type calcium channel modulation by PTHrP in bone 36

1.8 Specific objectives 37

CHAPTER II. MATERIALS AND METHODS

2.1 Cell preparation 41

2.2 Patch clamp technique 42

2.2.1 Solutions 42

2.2.2 Whole-cell and cell-attached recording 44

2.3 cAMP measurement 47

2.4 Drugs 47

2.5 Statistics 48

CHAPTER III. RESULTS - PART I: EFFECTS OF PTHrP AND PTH ON THE L-TYPE CALCIUM CHANNEL CURRENT IN UMR 106 CELLS

3.1 Introduction 50

3.2 Experimental Design 51

3.3 Results 51

3.3.1 Inward calcium currents 51

3.3.2 The L-type calcium channel current in UMR 106 cells 52

3.3.3 The single L-type calcium channel current in UMR 106 cells 52

3.3.4 Run-down of the calcium channel current in UMR 106 cells 53

3.3.5 Effect of PTHrP on the L-type calcium channel current 55

3.3.6 Time-course of the effect of PTHrP on the L-type calcium channel current 55

3.3.7 Effect of PTH on the L-type calcium channel current 56

3.3.8 Effect of PTHrP and PTH on the L-type calcium channel current in UMR 106 cells as a function of concentration 56

3.3.9 Effect of PTHrP on the steady-state activation and inactivation of the L-type calcium channel current in UMR 106 cells 57

3.3.10 The effect of PTHrP on single L-type calcium channels in UMR 106 cells 58

3.3.11 Open time distribution of single L-type calcium channels before and after the addition of PTHrP 59

3.4 Discussion 60

CHAPTER IV. RESULTS - PART II. SECOND MESSENGER MECHANISMS BY WHICH PTHrP MODULATES L-TYPE CALCIUM CHANNELS IN UMR 106 CELLS

4.1 Introduction 74

4.2 Experimental Design 75

4.3 Results 75

4.3.1 Effect of 8-bromo-cAMP on the L-type calcium channel current 75 UMR 106 cells 75

4.3.2 The extracellular application of Rp-cAMPs on the effect of PTHrP on the L-type calcium channel current in UMR 106 cells 76

4.3.3 Effect of forskolin on the L-type calcium channel current in UMR 106 cells 77

4.3.4 Effect of PTHrP and PTH on cAMP accumulation in UMR 106 cells 78

4.3.5 Effect of PTHrP and PTH, in the presence of IBMX, on intracellular cAMP accumulation in UMR 106 cells 79

4.3.6 Effect of 4B-phorbol 12 myristate 13-acetate (PMA) on the L-type calcium channel current in UMR 106 cells 79

4.3.7 The interaction between PMA and PTHrP on the L-type calcium channel current in UMR 106 cells 80

4.3.8 Effect of db-cGMP on the L-type calcium channel current in UMR 106 cells 81

4.4 Discussion 81

CHAPTER V. GENERAL DISCUSSION AND SUMMARY

5.1 General discussion 94

5.1.1 Effects of PTHrP and PTH on L-type calcium channel currents in UMR 106 cells 94

5.1.2 The investigation of second messenger mechanisms by which PTHrP modulates L-type calcium channel currents in UMR 106 cells 99

5.1.3 Clinical significance 107

5.2 Summary 108

5.3 Significance 110

References 112

LIST OF FIGURES

Fig. I-1. Schematic representation of the primary structure of mammalian PTH 38

Fig. I-2. Schematic representation of the primary structure of human PTHrP . 39

Fig. I-3. Schematic representation of PTH and/or PTHrP signal transduction . 40

Fig. III-1. The effects of Bay K 8644 and nifedipine on the L-type calcium channel current in a UMR 106 cell 63

Fig. III-2. The effects of Bay K 8644 and nifedipine on the L-type calcium channel current in a group of UMR 106 cells 64

Fig. III-3A. Current recordings from a single L-type calcium channel in a UMR 106 cell 65

Fig. III-3B. An all-points current amplitude histogram 65

Fig. III-3C. An I-V relationship of the single L-type calcium channel 65

Fig. III-4. Run-down of the L-type calcium channel current in UMR 106 cells . 66

Fig. III-5. The effect of PTHrP on the L-type calcium channel current in UMR 106 cells as a function of time 67

Fig. III-6. Peak inward current and the corresponding I-V relationship recorded from a UMR 106 cell before and after the addition of PTHrP 68

Fig. III-7. Peak inward current and the corresponding I-V relationship recorded from a UMR 106 cell before and after the addition of PTH 69

Fig. III-8A. The effect of 5 concentrations of PTHrP on the L-type calcium channel current recorded in UMR 106 cells 70

Fig. III-8B. The effect of 5 concentrations of PTH on the L-type calcium channel current recorded in UMR 106 cells 70

Fig. III-9A. Steady-state activation of the L-type calcium channel current in UMR 106 cells before and after the addition of PTHrP 71

Fig. III-9B. Steady-state inactivation of the L-type calcium channel current in UMR 106 cells before and after the addition of PTHrP 71

Fig. III-10A Single L-type calcium channel current recording from a UMR 106 cell 72

Fig. III-10B Single L-type calcium channel current recording from a UMR 106 cell after the addition of PTHrP 72

Fig. III-11A Open time distribution of the single L-type calcium channel current in a UMR 106 cell 73

Fig. III-11B Open time distribution of the single L-type calcium channel current after the addition of PTHrP in a UMR 106 cell 73

Fig. IV-1. The effect of 5 concentrations of 8-bromo-cAMP on the L-type calcium channel current in UMR 106 cells 86

Fig. IV-2. The effect of externally applied Rp-cAMPs, a PKA antagonist, on the PTHrP-induced increase of the L-type calcium channel current in UMR 106 cells 87

Fig. IV-3. The effect of forskolin, an adenylate cyclase agonist, on the L-type calcium channel current in UMR 106 cells 88

Fig. IV-4. The effect of PTHrP and PTH on intracellular cAMP accumulation in UMR 106 cells 89

Fig. IV-5. The effect of PTHrP and PTH, in the presence of IBMX, on cAMP accumulation in UMR 106 cells 90

Fig. IV-6. The effect of PMA, a PKC agonist, on the L-type calcium channel current in UMR 106 cells 91

Fig. IV-7. The effect of PMA, in the presence of PTHrP, on the L-type calcium channel current in a UMR 106 cell 92

Fig. IV-8. The effect of db-cGMP on the L-type calcium channel current in UMR 106 cells 93

Fig. V-1. Schematic representation of PTH-mediated activation of second messenger systems 111

Chapter I

Introduction and Literature Review

1.1. Introduction

PTHrP and PTH cause an increase in intracellular calcium concentration ($[Ca^{2+}]_i$) in osteoblasts. This, in turn, causes secretion of agents which activate osteoclasts resulting in bone resorption. The increase in $[Ca^{2+}]_i$ comes from two sources: intracellular stores and Ca^{2+} entry via plasma membrane Ca^{2+} channels. A major source of Ca^{2+} is via voltage-dependent L-type Ca^{2+} channels (Karpinski *et al.*, 1989; Yamaguchi *et al.*, 1989; Tsien *et al.*, 1990). In most cells, these channels are strongly regulated by second messenger pathways. Hence, it is important to determine how PTH regulates these channels during osteoblast activation. It has been demonstrated that L-type Ca^{2+} channels are activated by membrane depolarization. Studies have shown that PTH depolarizes osteoblast membranes (Ferrier *et al.*, 1988; Chesnoy-Marchais *et al.*, 1989), and this likely occurs through receptor-operated channels or second messenger-operated channels. After the L-type Ca^{2+} channels are activated, PTHrP and PTH may modulate their activity through second messenger pathways yet to be elucidated. The following literature review discusses the physiological roles of PTHrP and PTH, particularly in bone physiology.

1.2. The discovery and history of PTH and PTHrP

The importance of parathyroid hormone (PTH) in normal calcium (Ca^{2+}) homeostasis resulting from its effects on bone has long been recognized. A second member

of the parathyroid hormone family, parathyroid hormone-related peptide (PTHrP), binds to the same receptor as parathyroid hormone, and is thought to have similar effects on bone. The isolation of PTH was accomplished independently by two scientists: Adolph M. Hanson in 1923 and James B. Collip in 1925 (Eknoyan, 1995). After parathyroid extract became available for research, several investigators, such as Fuller Albright, Franklin C. McLean, Harvey M. Patt, and Arno B. Luckhardt, were able to demonstrate the homeostatic effects of PTH on bone and kidney. The final purification of bovine PTH for sequence analysis was published by Brewer and Ronan, as well as Niall *et al.* in 1970 (Eknoyan *et al.*, 1995). Since then, sequences for PTH from a number of different species have been identified (eg. human, bovine, and porcine). These PTH molecules all have similar primary structures and a molecular weight of approximately 9.6 kD (Eknoyan *et al.*, 1992). Early experiments demonstrated that most of the biological activity of PTH exists within the first 34 residues of the (N)-terminus, and it is thought that the carboxy (C)-terminus functions to extend the half-life of the molecule in the circulation. PTH is essential for Ca^{2+} homeostasis, as it has both catabolic and anabolic effects on the skeleton. Many studies are being performed to investigate the mechanisms of PTH action, particularly on bone, in order to target possible therapeutic roles for PTH in the treatment of osteoporosis.

Parathyroid hormone-related peptide (PTHrP), which binds to the same receptors as PTH, was discovered in 1987. Although it is not thought to play a significant role in daily Ca^{2+} homeostasis, it is found extensively in tissues throughout the body in both fetal and adult life. PTHrP was discovered simultaneously and independently by three

laboratories (Philbrick *et al.*, 1996). It was identified during the search for the humoral agent responsible for humoral hypercalcemia of malignancy (HHM) (Philbrick *et al.*, 1996). Hypercalcemia was first described in patients with cancer in the 1920's. In 1941, Albright speculated that a circulating calcemic factor was being secreted by the carcinoma and that the hypercalcemia was "humoral" or endocrine in nature. He thought that the humoral factor was PTH or a related substance. In 1956, Plimpton and Gelhorn, and Connor and Thomas reported two series of patients with cancer in whom hypercalcemia reversed after tumor eradication. It is now known that there are two common forms of malignancy-associated hypercalcemia. One form is described as local osteolytic hypercalcemia as a result of invasion of the skeleton by malignant cells, usually in the form of myeloma, leukemia, lymphoma, or breast cancer; this form occurs in approximately 20% of the patients with malignancy-associated hypercalcemia. The majority of the remaining 80% falls into the humoral hypercalcemia of malignancy category, which was characterized by Albright *et al.* (1941). These patients usually have squamous cell carcinomas (most often lung, esophagus, cervix, head, and neck), renal carcinomas, bladder carcinomas, certain lymphomas, and breast carcinomas. In these cases, there are no skeletal metastases, and the hypercalcemia is reversible with tumor excision, confirming Albright's "humoral hypothesis". HHM patients displayed an increased rate of osteoclastic bone resorption, but a decreased rate of osteoblastic bone formation. By 1980, HHM had been well defined in biochemical terms, however, the humoral agent being secreted from the carcinoma had not yet been identified. In 1987 three groups described the purification and NH_2-terminal amino acid sequence of PTHrP and the molecular cloning of corres-

ponding complementary DNAs. By the early 1990's human, rat, mouse, and chicken PTHrP genes had been identified and described in structural terms. It is now established that the most common form of HHM is caused by PTHrP. It is also confirmed that in patients with HHM, PTHrP is secreted into the systemic circulation in classic endocrine fashion by the tumor in question, and it interacts with PTH receptors in the skeleton and kidney.

Since the discovery of PTHrP, many researchers have focused on the normal physiological functions of PTHrP (Watson *et al.*, 1992). It is now apparent that the PTHrP gene is expressed in essentially every tissue and organ of the body at some point in life (Watson *et al.*, 1992). The importance of PTHrP was confirmed when damage to the PTHrP gene was shown to be, in most cases, lethal (Watson *et al.*, 1992).

1.3. The structure of PTH and PTHrP

After extensive processing, the final PTH product is derived from the sequential enzymatic cleavage of a precursor gene product, preproPTH, consisting of 115 amino acids. PreproPTH is cleaved to proPTH (90 amino acids) and the final active PTH consists of 84 amino acids (Civitelli *et al.*, 1995) (see Fig.I-1). The 34 amino acids of the amino terminus of the active hormone are essential for the normal activity of PTH. Step-wise shortening of PTH from position 34 toward position 25 results in a progressive decline in potency. The 1-34 region of the PTH molecule is subdivided into a short activation domain (amino acids 1-6) and a longer receptor binding domain (amino acids 7-34). Amino acids 25-31 in the PTH molecule display the minimal structural requirements for detectable

receptor occupancy (Civitelli *et al.*, 1995).

PTHrP consists of 3 isoforms with distinct COOH terminals (Philbrick *et al.*, 1996). Each of the 3 isoforms contains a common 36 amino acid "prepro" or signal sequence (Philbrick *et al*, 1996). In addition, each of the isoforms contains 139 amino acids in common (Philbrick *et al.*, 1996). One of the isoforms terminates at amino acid 139, another extends to amino acid 141, and the third extends to 173 amino acids (Philbrick *et al.*, 1996). PTHrP shares a homologous region with PTH, which is amino acids 1 through 13 (Philbrick *et al.*, 1996) (see Fig. I-2). Despite the fact that amino acids 14-34 of PTHrP share no sequence homology with PTH, they are highly similar in conformational terms (Philbrick *et al.*, 1996). Thus, the region of PTHrP that is similar to PTH extends from amino acid 1 to 34. It has been suggested that this is why PTHrP and PTH are able to bind to the same receptor on target cells. At present, the exact structure of the secretory form of PTHrP remains unknown (Civitelli *et al.*, 1995).

1.4. Synthesis and secretion of PTH and PTHrP

PTH is primarily regulated by serum Ca^{2+} levels, however, its synthesis and secretion are also governed by vitamin D, steroid hormones, and neurotransmitters (Watson *et al.*, 1992). Ca^{2+} regulates PTH secretion by increasing the release of PTH from the parathyroid gland when serum Ca^{2+} levels are low. Several studies suggest that the parathyroid gland has two separate secretory pools of PTH, one composed of stored secretory granules, which respond to agents which elevate intracellular cAMP ([cAMP]$_i$) levels, and the second composed of newly synthesized PTH, which responds directly to

extracellular Ca^{2+} levels. The parathyroid gland is thought to detect levels of extracellular Ca^{2+} due to Ca^{2+} receptors on the parathyroid membrane. It has been suggested that the binding of Ca^{2+} to specific parathyroid Ca^{2+} receptors activates traditional second messenger pathways in the cells of the parathyroid gland, thereby increasing or decreasing PTH secretion (Watson *et al.*, 1992). Not only does Ca^{2+} regulate the release of PTH from the parathyroid gland, but Ca^{2+} also controls the intraparathyroid degradation of intact PTH to peptide fragments, some of which are secreted. Recent studies have implicated calpains, which are calcium-dependent proteases, as the enzymes responsible for PTH degradation (Watson *et al.*, 1992). It is likely that this intracellular degradation of PTH to peptide fragments accounts for the ability of the parathyroid gland to decrease the availability of intact PTH in the event of a rapid rise in serum Ca^{2+} (Watson *et al.*, 1992). Blood Ca^{2+} levels also regulate PTH synthesis in the parathyroid gland. At normal levels of extracellular Ca^{2+}, the parathyroid synthesizes PTH at a nearly maximal rate (Watson *et al.*, 1992).

Since PTH stimulates renal production of 1,25 dihydroxyvitamin D, investigators have searched for a negative feedback effect of vitamin D metabolites on the parathyroid gland (Watson *et al.*, 1992). Recent studies have demonstrated the suppression of PTH release by 1,25 dihydroxyvitamin D through inhibition of PTH gene expression (Watson *et al.*, 1992).

Glucocorticoids, such as cortisol, have the ability to enhance the release of PTH (Watson *et al.*, 1992). Treatment of abnormal parathyroid cells with other steroid hormones, such as estradiol and progesterone, also increases the release of PTH. It has

also been found that estradiol deficiency, which occurs in many post-menopausal women, causes a shift in the normal amount of plasma Ca^{2+} in the blood required to suppress PTH secretion; thus, a higher serum Ca^{2+} concentration is needed to suppress secretion of PTH (Watson *et al.*, 1992).

It is well documented that several neurotransmitters have the ability to affect PTH release, but the physiological importance of these neurotransmitters in PTH secretion is not known (Watson *et al.*, 1992). For instance, dopamine and β-agonists cause a prompt, but short-lasting release of PTH (Watson *et al.*, 1992). In addition, the presence of neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P in nerves surrounding the parathyroid gland suggest a role for these substances in PTH secretion (Watson *et al.*, 1992).

The actions of PTHrP differ in many ways from those of PTH. For example, PTH is a "traditional" hormone acting on target sites distant from its site of secretion. In contrast, PTHrP is thought to act mainly in an autocrine or paracrine manner; this is consistent with the significantly lower levels of PTHrP in the circulation (Civitelli *et al.*, 1995). Although PTHrP is a newly-discovered molecule, extensive studies are uncovering its significance in normal physiology. It is distributed in tissues throughout the body and the regulation of its secretion appears to be governed by the microenvironment of the tissue (Civitelli *et al.*, 1995). Much interest has focused on the factors that regulate PTHrP expression, and studies have shown a number of agents that directly affect the level of gene transcription. For instance, glucocorticoids are the most well known agents that down-regulate PTHrP expression and decrease mRNA and protein production by approximately

70%. (Lu *et al.*, 1989, Ikeda *et al.*, 1989, and Glatz *et al.*, 1994). This is of importance, since several hypercalcemic disorders, such as HHM, are treated with glucocorticoids. Another agent that down-regulates PTHrP expression is testosterone (Liu *et al.*, 1993) . On the other hand, there are several substances that up-regulate PTHrP expression, such as angiotensin II (Pirola *et al.*, 1993), cAMP (Zajac *et al.*, 1989, Chilco *et al.*, 1993, Ikeda *et al.*, 1993, and Chan *et al.*, 1990), cyclohexamide (Kiriyama *et al.*, 1993), EGF (Gillespie *et al.*, 1992), TGF-β (Gillespie *et al.*, 1992), estradiol (Gillespie *et al.*, 1992, Suva *et al.*, 1991), prolactin (Thiede, 1989), sodium butyrate (Streutker *et al.*, 1991), interleukin 2 (Ikeda *et al.*, 1993), and retinoic acid (Chan *et al.*, 1990). So far, no direct evidence of an effect of Ca^{2+} on gene expression has been demonstrated, but there is growing speculation for an effect of Ca^{2+} on regulating PTHrP secretion (Zajac *et al.*, 1989, Ikeda *et al.*, 1989, and Hellman *et al.*, 1992).

Even though levels of PTHrP in the blood are usually low, studies have attempted to identify the circulating forms of PTHrP. It has been exceptionally difficult, however, to identify the circulating forms of this hormone in healthy individuals. Although N-terminal regions of PTHrP cannot be detected in the circulation of normal individuals, in individuals with HHM, N-terminal fragments of PTHrP are easily detectable. On the other hand, there is evidence of a circulating C-terminal fragment of PTHrP in individuals without HHM, since in patients with renal failure, there is the presence of C-terminal epitopes, and the absence of N-terminal epitopes in the blood. (Moseley *et al.*, 1995). It may be that the C-terminal fragment of PTHrP is removed by the kidney, which is why it is detectable in the blood following renal failure. In addition, the detection of C-terminal

PTHrP in normal urine supports this notion (Moseley *et al.*, 1995). The source of the PTHrP and the size of the fragment released into the circulation are not known. The detection of N-terminal fragments in HHM patients and C-terminal fragments in patients with renal failure was performed by radioimmunoassays. However, in addition to these radioimmunoassays, gel filtration and immunoextraction studies suggest that separate peptides containing the N- and C-terminal epitopes are present in the circulation. A recent study found that in patients with HHM, levels of PTHrP (37-84) were ninefold higher than levels of PTHrP (1-74) (Burtis *et al.*, 1994). This provides strong evidence that in addition to the N-terminal fragment (1-34) found in HHM patients, a major circulating form is a mid-molecule fragment from which the N-terminal portion has been cleaved. It has been suggested that multiple fragments of PTHrP will eventually be identified in the circulation.

1.5. Physiological actions of PTH and PTHrP

PTH helps to maintain Ca^{2+} homeostasis by regulating the circulating concentration of ionized Ca^{2+} through key actions on target organs (Dempster *et al.*, 1993). The parathyroid gland is extremely sensitive to any perturbation of Ca^{2+}, and responds with an increase in PTH secretion if Ca^{2+} declines, and a decrease in PTH secretion if Ca^{2+} increases (Dempster *et al.*, 1993). The dominant form of active PTH is the entire molecule (1-84). Normally, 70-95% of circulating PTH is present in inactive C-terminal fragments, whereas intact active PTH represents only 5-30% of circulating forms (Kronenberg *et al.*, 1996). N-terminal fragments have not been found in the circulation in appreciable quantities. The half-life of PTH (1-84) in the blood is less than 3 min. This rapid

metabolism of PTH (1-84) ensures that the availability of PTH for receptor binding is directly regulated by the secretory rate of PTH from the parathyroid gland in response to minute-to-minute fluctuations in blood Ca^{2+} concentrations (Fitzpatrick *et al.*, 1996). PTH regulates levels of blood Ca^{2+} primarily through its bone resorptive effect. An additional major target organ for PTH is the kidney, as PTH increases phosphate excretion and Ca^{2+} uptake. The heart, vascular smooth muscle and the gastrointestinal tract are also targets for PTH action (Fitzpatrick *et al.*, 1992). PTH maintains Ca^{2+} homeostasis, despite moderate fluctuations in diet, bone metabolism and renal function (Kronenberg *et al.*, 1996).

In contrast to PTH, PTHrP probably does not play a major role in daily Ca^{2+} homeostasis; however, since PTHrP is found extensively throughout tissues, it has been suggested that it plays a vital role in development and in normal physiology, particularly a local one at the tissue or cell level (Moseley *et al.*, 1996). For instance, PTHrP has PTH-like actions on bone and kidney: it also stimulates bone resorption and decreases phosphate reabsorption in the kidney. In addition to bone and kidney, PTHrP has been found in a wide range of tissues including brain, skin, endocrine tissues, lung, gut, muscle, heart, breast, and breast milk (Moseley *et al.*, 1996). Furthermore, PTHrP has been implicated in fetal development and in lactation and is thought to act as a cytokine in several physiological processes. Because there is no convincing evidence that PTHrP circulates in the normal nonpregnant adult, it is probable that PTHrP has an autocrine or paracrine function in most normal tissues (Moseley *et al.*, 1996). The actions of PTHrP are being studied in an attempt to delineate its role in normal physiology.

1.5.1. Cellular actions of PTH

1.5.1.1. Cellular actions of PTH on Bone

PTH can have both catabolic and anabolic effects on the skeleton, depending upon the dose and the period of time that bone is exposed to PTH. For instance, the catabolic effects of PTH on bone are seen at high doses and upon brief exposure, whereas, the anabolic effects of PTH are evident at low doses and upon long exposure.

Many studies have investigated the catabolic effects of PTH on bone and the physiological process of bone resorption mediated by the hormone. PTH indirectly activates osteoclasts, the bone cells responsible for bone resorption. There are two known phases of PTH-induced bone resorption (Fitzpatrick *et al.*, 1992). The first phase is a rapid response to PTH in which cells lining the endosteal surfaces of bone release Ca^{2+} in less than 1 hour. This first phase of PTH action is associated with increased metabolic activity of osteoclasts. The second phase of Ca^{2+} mobilization depends upon protein synthesis and occurs within 24 hrs of PTH administration. In this phase, there is an increase in both the number and metabolic activity of osteoclasts. PTH is known to have direct effects on the osteoblasts, the cells responsible for bone formation; however, there has been little evidence for any direct action of PTH on osteoclasts. PTH acts through second messenger systems, such as adenylate cyclase and phospholipase C in osteoblasts (Civitelli *et al.*, 1995). It also increases L-type Ca^{2+} channel currents in neonatal rat calvaria (Fritsch *et al.*, 1994). These results suggest that the osteoblast is a major target cell for PTH.

Studies have attempted to uncover the mechanism by which PTH indirectly activates osteoclast bone resorption through its actions on osteoblasts. It has been

speculated that a signal generated by the osteoblast, such as the release of cytokines, activates the osteoclast. Interleukin-6, produced by osteoblasts in response to PTH, may be one messenger (Feyen *et al.*, 1989). Insulin-like growth factor 1(IGF-1) and insulin-like growth factor 2 (IGF-2) have also been implicated in this coupling event (Linkhart *et al.*, 1989; Canalis *et al.*, 1989).

Although PTH stimulates osteoclast-mediated bone resorption, the exact mechanism of this process is not fully understood. Bone resorption is thought to occur by enzyme release by osteoclasts. For instance, acid phosphatases and carbonic anhydrase are two substances that are speculated to be secreted from osteoclasts which create an acidic environment that is necessary for bone resorption to occur (Fitzpatrick *et al.*, 1992).

In addition to its catabolic effects, PTH also has anabolic actions on osteoblasts. Under normal physiological conditions, PTH plays a significant role in bone remodelling, which suggests its involvement in bone formation in addition to bone resorption. PTH stimulates the formation of prostaglandin E_2 in cultured mouse calvarial bone (Ljunggren *et al.*, 1989), which mediates an increase in osteoblast precursor replication, and subsequently the stimulation of bone formation. The *in vivo* anabolic actions of PTH have been demonstrated following low-dose administration, which leads to increases in bone mineral density (Slovik *et al.*, 1986). It is apparent that the effect of PTH on bone depends upon the dose and the time of exposure, with lower doses and intermittent administration leading to anabolic effects and higher doses and long-term exposure leading to bone resorption.

The cellular actions of PTH on bone are extensive and consist of both well

documented and newly discovered effects.

1.5.1.2. Cellular actions of PTH on the kidney

The kidney, like bone, is a major target organ for PTH (Fitzpatrick *et al.*, 1992). PTH is a phosphaturic hormone, as it prevents the kidneys from reabsorbing filtered phosphate. This inhibition occurs primarily in the proximal convoluted tubule and pars recta (Agus *et al.*, 1981), but it may also be inhibited in the distal tubule (Fitzpatrick *et al.*, 1992). In addition, PTH stimulates Ca^{2+} reabsorption from the glomerular fluid. This helps to maintain serum Ca^{2+} levels when they are low.

Another major action of PTH on the kidney is the stimulation of 1,25-dihydroxyvitamin D formation (Fitzpatrick *et al.*, 1992). The enzyme responsible for the conversion of 25-hydroxyvitamin D to 1,25-dihydroxyvitamin D, renal 1 alpha-hydroxylase, is located in the proximal tubule (Kawaskima *et al.*, 1981). PTH stimulates the activation of this enzyme (Slovik *et al.*, 1984). PTH, therefore, indirectly stimulates Ca^{2+} absorption in the gastrointestinal tract, an action that is directly stimulated by 1,25-dihydroxyvitamin D.

1.5.1.3. Cellular actions of PTH on the cardiovascular system

The effect of PTH on the heart and vasculature has been extensively studied. It has been established that PTH is a vasoactive substance that is important in regulating vascular tone (Mok *et al.*, 1989). Pang *et al.* (1988) suggested that PTH may exert its action on blood vessels by inhibiting L-type Ca^{2+} channels. Wang *et al.* (1991) confirmed these

findings by also showing that bPTH (1-34) exerts its vasorelaxing effect on vascular smooth muscle through its action on the L-type Ca^{2+} channel.

In addition to its vascular dilatory properties, PTH has been implicated to have major effects on the heart (Dipette *et al.*, 1992). For instance, it may increase heart rate, coronary blood flow, and contractility (Bogin *et al.*, 1981; Chui *et al.*, 1991, Dipette *et al.*, 1992; Roca-Cusachs *et al.*, 1991, Tenner *et al.*, 1983). Wang *et al.* (1991) and Rampe *et al.* (1991) demonstrated that PTH exerts inotropic effects on neonatal rat ventricular myocytes by increasing the L-type Ca^{2+} channel current.

1.5.2. Cellular actions of PTHrP

1.5.2.1. Cellular actions of PTHrP on bone

This hormone has several actions on bone, both physiological and pathological. PTHrP has some effects on bone that are similar to those of PTH, but it also has some novel actions. PTHrP (1-34), (1-84), and (1-141), like PTH (1-34), stimulates bone resorption in organ culture and by isolated osteoclasts in the presence of osteoblasts (Hammonds *et al.*, 1989, Horiuchi *et al.*, 1987). In cocultures, radiolabelled PTHrP, like PTH, binds only to osteoblasts and affects osteoclastic bone resorption indirectly (Evely *et al.*, 1991).

Although both hormones stimulate bone resorption, contradictory results have been reported regarding the potencies of PTHrP and PTH. For instance, in a study by Fukayama *et al.* (1989), human PTHrP was found to be less potent than human PTH in stimulating bone resorption in human bone cells (Yamaguchi *et al.*, 1989b). On the otherhand, PTHrP

has been found to have a greater *in vivo* hypercalcemic effect than PTH in rat bone cells (Horiuchi *et al.*, 1987). Moreover, PTHrP and PTH were found to be equally potent in stimulating bone resorption in rats (Stewart *et al.*, 1988). One possible explanation for this inconsistency in potencies among PTHrP and PTH is that intracellular pathway activation between the two hormones may be different. Further studies would have to be performed to investigate the intracellular mechanism of action of PTHrP and PTH in osteoblasts.

PTHrP has some actions on bone that differ from those of PTH. PTHrP is the agent responsible for humoral hypercalcemia of malignancy (HHM). One of the characteristic features of HHM is the uncoupling of bone resorption and bone formation, with bone resorption being enhanced. Moseley *et al.* (1995) found when nude mice transfected with the HHM gene were treated with an antibody against the N-terminal portion of PTHrP, a decrease in the hypercalcemia resulted. In addition, a reversal of the bone abnormalities occurred. The precise mechanism of PTHrP to decrease bone formation is unknown.

In addition to its effect on osteoblasts, PTHrP has been found to have an effect on osteoclasts, with the 107-111 domain being a potent inhibitor of osteoclastic bone resorbing activity *in vitro* (Fenton *et al.*, 1991, Fenton *et al.*, 1991 and Fenton *et al.*, 1993). PTHrP (107-111) has been shown to inhibit bone resorption *in vitro* in isolated osteoclasts, however, it has not been shown to inhibit bone resorption in fetal long bone or mouse calvaria (Sone *et al.*, 1992). The physiological relevance of this fragment of PTHrP is currently an area of controversy. It is possible that this action of PTHrP could be relevant during fetal bone development in which bone formation, rather than bone resorption, predominates (Moseley *et al.*, 1995).

Although evidence suggests a role of PTHrP in normal fetal bone physiology, the precise relevance of PTHrP in adult human bone is not clear. The presence of PTHrP in adult human bone would indicate a role in bone turnover, but further studies would have to be performed to confirm this hypothesis (Moseley *et al.*, 1995).

1.5.2.2. Cellular actions of PTHrP on the kidney

In normal individuals, PTHrP and PTH have similar effects on the kidney. For instance, in most *in vivo* models, PTHrP (1-34) causes increased 1,25-dihydroxyvitamin D levels, as does PTH. There have been several studies that report effects of PTHrP on the kidney in patients with HHM (Moseley *et al.*, 1995). In these patients, renal conservation of Ca^{2+} is an important factor in the development of hypercalcemia (Moseley *et al.*, 1995).

1.5.2.3. The role of PTHrP in fetal development

PTHrP has been implicated in fetal development. PTHrP, as well as PTHrP/PTH receptors, have been detected in several fetal tissues throughout development. PTHrP exhibits a temporal pattern of expression in tissues (Campos *et al.*, 1991; Dunne *et al.*, 1994; Moniz *et al.*, 1990; Moseley *et al.*, 1991). The expression of PTHrP and PTHrP/PTH receptors in fetal tissues, placental cells, placental membranes, and amniotic fluid (Bowden *et al.*, 1994; Dunne *et al.*, 1994; Dvir *et al.*, 1995; Fergusson *et al.*, 1992, Senior *et al.*, 1991; Wlodek *et al.*, 1995) during uterus implantation (Becke *et al.*, 1993) and throughout gestation (Thiede *et al.*, 1990; Williams *et al.*, 1994) suggests the

likelihood of important roles for PTHrP in normal fetal development. In addition, PTHrP has been found to affect the uterus by having a relaxant effect (Thiede *et al.*, 1990). PTHrP is also thought to be a major regulator of placental Ca^{2+} transport (Abbas *et al.*, 1989; Rodda *et al.*, 1988).

1.5.2.4. The role of PTHrP in lactation

PTHrP has also been implicated to play a role in lactation. For instance, it has been found in breast tissue from pregnant (Rakopoulos *et al.*, 1992) and lactating women (Thiede *et al.*, 1988) and also in milk (Budayr *et al.*, 1989; Law *et al.*, 1991; Ratcliffe *et al*, 1992). Release of PTHrP is increased by suckling (Thiede *et al.*, 1988) and by prolactin (Thiede, 1989), indicating a role in milk production. Particularly high levels of PTHrP are found in breast milk; however, it is not known whether it is involved in milk formation or in the transport of Ca^{2+} into milk, or whether it serves some other purpose. Significant levels of PTHrP have been found in the circulation of lactating women (Dobnig *et al.*, 1995; Grill *et al.*, 1992; Kovacs *et al.*, 1995) where it may have an endocrine role in mobilizing maternal bone Ca^{2+} for milk production. Growing evidence suggests a role for PTHrP in mammary cell growth, as PTHrP is temporally expressed in mammary tissue during pregnancy and lactation (Khosla *et al.*, 1990; Rakopoulos *et al.*, 1992).

1.5.2.5. The role of PTHrP as a cytokine

PTHrP is extensively distributed in fetal tissues (Campos *et al.*, 1991; Dunne *et al.*, 1994; Moseley *et al.*, 1991; Senior *et al.*, 1991), particularly in the epithelia, and in adult

skin (Hayman *et al.*, 1989; Kremer *et al.*, 1991) It has been suggested that PTHrP acts as a cytokine involved in cell growth and differentiation. PTHrP is likely a paracrine regulator of epithelial growth and differentiation (Moseley *et al.*, 1991). PTHrP may also function in $[Ca^{2+}]_i$ control and cell cycle events. Since PTHrP is expressed in many adult tissues, and in developing fetal tissues in which it can be localized to ectodermal, mesodermal, and endodermal tissue (Dunne *et al.*, 1994; Moseley *et al.*, 1991; Senior *et al.*, 1991), it is suggested that PTHrP functions in cell growth and differentiation in these cells.

Cultured keratinocytes, which synthesize and secrete PTHrP, have been used to examine the role that PTHrP plays in cell growth (Kremer *et al.*, 1991). Holick and coworkers (1994) found that proliferation of cultured keratinocytes was inhibited in the presence of PTHrP, an effect mediated by the PTH-like portion of the molecule. In addition to inhibiting keratinocyte growth, PTHrP has been shown to promote differentiation of keratinocytes (Kaiser *et al.*, 1994).

1.5.2.6. The role of PTHrP in the cardiovascular system

PTHrP is a potent vasodilator of arteries and arterioles. The discovery that PTHrP is a regulator of smooth muscle and cardiac muscle tone is based on the fact that PTHrP is expressed at sites adjacent to smooth muscle beds and that PTHrP (1-34) causes relaxation of smooth and cardiac muscle. PTHrP can relax smooth muscle in blood vessels (Crass *et al.*, 1993), in the uterus (Shew *et al.*, 1991), and in the gastrointestinal tract (Mok *et al.*,1989). In addition, expression of PTHrP increases with stretch of the urinary

bladder (Yamamoto *et al.*, 1992), uterus (Thiede *et al.*, 1990), and aorta (Pirola *et al.*, 1994).

Like PTH, PTHrP has been shown to have chronotropic and inotropic actions in cultured heart cells. In rat atria, PTHrP increased both the rate of contraction and the force of contraction in a concentration-dependent manner (Thiede *et al.*, 1995). In addition, Nickols *et al.* (1989) observed that PTHrP produced rapid and dose-related increases in heart rate and contractility.

1.6. Actions of PTHrP and PTH on intracellular signalling systems

The existence of a PTHrP/PTH receptor in most tissues, including bone, which binds both PTHrP and PTH, has been known for some time. It is currently known that upon binding of these hormones to the PTHrP/PTH receptor, both the adenylate cyclase and PLC signal transduction pathways are activated (Abou-Samra *et al.*, 1992). However, there is still controversy about the intracellular mechanisms of action of PTHrP and PTH, since several studies have reported conflicting results. Since PTHrP and PTH play important roles in bone resorption, it is of interest that the intracellular pathways for these hormones be elucidated. This section is an overview of the findings of several investigators studying the signalling pathways which are activated by PTHrP or PTH in a variety of bone cell lines.

It has been reported that PTHrP and PTH activate both the adenylate cyclase pathway and the phospholipase C pathway in several bone cell lines (Morris *et al.*, 1996) (see Fig. I-3). Activation of the adenylate cyclase pathway leads to the generation of

cAMP, whereas activation of the phospholipase C pathway leads to the generation of inositol phosphates (IPs) and diacylglycerol (DAG) (Morris *et al.*, 1996). The PTHrP/PTH receptor is associated with a number of different G proteins (depending upon which pathway is activated) which consist of an α subunit and a lipophilic βγ subunit (Spiegel *et al.*, 1994). When a ligand binds to the PTHrP/PTH receptor, leading to activation of the inactive αβγ heterotrimeric complex, the GDP bound to the α subunit is replaced with GTP. The GTP-α subunit separates from the βγ subunit and activates the catalytic unit of the signal transduction complex associated with the G protein. There are multiple G proteins, each of which is characterized by a unique amino acid sequence of the α unit. In the case of adenylate cyclase activation, the associated G protein is Gs. Activation of Gi proteins usually results in the inhibition of the adenylate cyclase pathway (Morris *et al.*, 1996). The G proteins that are associated with the activation of the phospholipase C pathway include those whose ability to activate PLC are inhibited by pertussis toxin and those that are insensitive to pertussis toxin inhibition (Gq) (Morris *et al.*, 1996). The inactivation of the G protein results when bound GTP is hydrolyzed to GDP by the action of an endogenous GTPase activity of the α subunit, followed by the rejoining of the α subunit to the β and γ units. This activation-deactivation cycle of the G protein ensures that persistent activation by a particular ligand does not occur.

Constant stimulation of messenger pathways can be prevented by the G protein activation-inactivation process and by receptor desensitization (Bidwell *et al.*, 1991). The latter involves the reduced responsiveness of the receptor to PTHrP or PTH stimulation. Although the exact mechanism of this homologous desensitization is not known for the

PTHrP/PTH pathway, possibilities include phosphorylation of the PTHrP/PTH receptor, uncoupling of the receptor from the G protein (Fukayama *et al.*, 1992), sequestration, and loss of the PTHrP/PTH receptor by an internalization process (Huang *et al.*, 1994).

Although both signal transduction pathways (ie. adenylate cyclase and PLC) have been found to be activated by PTHrP and PTH, the pathway which dominates, or the way in which these pathways interact remains controversial. In addition, the multitude of PTHrP and PTH fragments that exist have no apparent function to regulate one specific pathway or the other *in vivo* (Murray *et al.*, 1994). Although the PTHrP/PTH receptor appears to lack specificity for the adenylate cyclase and PLC pathways, it is established that the regulation of the adenylate cyclase and phospholipase C pathways by PTHrP and PTH is complex, and varies from tissue to tissue. Since PTHrP and PTH are both known to increase $[Ca^{2+}]_i$ in several osteoblast cell lines, and this has been suggested to stimulate osteoblasts to signal the osteoclasts to initiate bone resorption, discovering the means by which the increase in Ca^{2+} occurs is significant. One way in which $[Ca^{2+}]_i$ increases is by influx of Ca^{2+} through voltage-dependent Ca^{2+} channels in the plasma membrane. PTHrP may regulate these channels to allow an influx of Ca^{2+} by activating the following pathways: 1) adenylate cyclase pathway (by protein kinase A activation and subsequent phosphorylation of membrane Ca^{2+} channels). 2) phospholipase C pathway (by protein kinase C activation and subsequent phosphorylation of membrane Ca^{2+} channels, or by the splitting of phosphoinositides, such as PIP_2 into IP_3 and diacylglycerol, the former of which stimulates the release of $[Ca^{2+}]_i$ stores). 3) guanylate cyclase pathway (cGMP/PKG). 4) A G protein that is directly linked to membrane Ca^{2+} channels. 5) an unknown signal

transduction pathway.

1.6.1. PTH and PTHrP receptors

Although the structural homology between PTHrP and PTH is limited, they have very similar physiological actions, and many investigators believe that the two hormones bind to the same receptor on target cells, such as bone. (Juppner, H. *et al.*, 1988). Recently, this assumption was confirmed in a study by Schipani *et al.* (1993) in which they were able to clone the gene that encodes the receptor that binds both PTHrP and PTH. The PTHrP/PTH receptor is G-protein linked and its tertiary structure resembles that of β-adrenergic receptors. The PTHrP/PTH receptor is monomeric, with 7 transmembrane domains, an extracellular N-terminus, and a long C-terminus tail (Schipani *et al.*, 1993). This receptor binds PTH and the PTH-like region of PTHrP (1-34).

Recent pharmacological studies indicate that the affinity of PTHrP and PTH fragments for this receptor differs from tissue to tissue. For instance, certain PTH fragments were found to bind to bovine renal membranes with a higher affinity than to rat osteosarcoma cells (Juppner et. al., 1988), however, the opposite was found with intact PTH (Demay *et al.*, 1985). In addition, PTHrP peptides had different affinities for the receptors on membranes of different species. This would suggest the possibility of organ-specific receptors that have different affinities for a variety of ligands and /or that the binding of these ligands may activate different signal transduction pathways. (Juppner *et al.*, 1988).

It was thought that there were two types of PTH receptors, each of which activated

separate signal transduction pathways. It is now known that both the adenylate cyclase pathway and the phospholipase C pathway are coupled to the same PTHrP/PTH receptor (Abou-Samra *et al.*, 1992). It has been documented that discrete PTHrP and PTH fragments as well as specific domains of the PTHrP/PTH receptor preferentially activate one or the other pathway. In addition, the primary pathway activated by PTHrP and/or PTH varies from tissue to tissue or among different cell lines. Although this dual signal transduction system has been confirmed for the PTHrP/PTH receptor, there is still a controversy about the possibility of a second PTH receptor which may be activated by the C-terminal regions of the PTH molecule.

In addition to the PTHrP/PTH receptor, there is a receptor specifically for PTHrP that binds the midregion and COOH portion of the peptide that is dissimilar to PTH. Although most of the effects of PTHrP occur after binding to the PTHrP/PTH receptor, this PTHrP-specific receptor has been localized in pancreatic β-cells, in lymphocytes, and in keratinocytes; it appears to signal through the protein kinase C pathway, not through adenylate cyclase. The structure of this receptor remains unclear (Philbrick *et al.*, 1996).

1.6.2. Effects of PTHrP and PTH on intracellular calcium concentration

It is known that Ca^{2+} serves as a second messenger in many types of cells, including osteoblasts. An increase in $[Ca^{2+}]_i$ may occur due to either a release of Ca^{2+} from intracellular stores or by an influx of Ca^{2+} from the extracellular fluid through either voltage-activated Ca^{2+} channels or receptor-operated Ca^{2+} channels; these channels may be activated by the adenylate cyclase and/or the phospholipase C pathways and/or G

proteins directly linked to the channels. It is established that PTHrP/PTH receptors do not exist on osteoclasts, are present on osteoblasts, which indicates a role for $[Ca^{2+}]_i$ in stimulating bone resorption indirectly through an action on osteoblasts.

PTHrP and PTH are both known to increase $[Ca^{2+}]_i$, after binding to the PTHrP/PTH receptor in osteoblasts. Many studies have been performed to determine the intracellular biochemical pathway(s) which is (are) activated to cause the rise in $[Ca^{2+}]_i$. Yamaguchi *et al.* (1987a) described a triphasic $[Ca^{2+}]_i$ increase in UMR-106 cells, an osteoblast-like cell line from rats. Phases one and two were characterized by a rapid transient increase in $[Ca^{2+}]_i$, followed by a rapid decrease in $[Ca^{2+}]_i$, whereas phase three was described as a slower, more prolonged increase in $[Ca^{2+}]_i$. Furthermore, phase one channels were sensitive to Ca^{2+} channel blockers, such as verapamil, whereas the phase three channels were not. In addition, the phase three channel appeared to be modulated by cAMP, since forskolin stimulation of adenylate cyclase produced a phase three Ca^{2+} transient, but not a phase one Ca^{2+} transient. Phase two was characterized by a maintained rapid increase in $[Ca^{2+}]_i$ followed by a rapid decrease in $[Ca^{2+}]_i$, sometimes below control levels. The differences among these phases strongly suggest that PTH activates two independent Ca^{2+} channels which are stimulated by different mechanisms (Yamaguchi *et al.*, 1987a).

Yamaguchi *et al.* (1987a) were also able to determine that the rapid transient increase in $[Ca^{2+}]_i$, which they termed phases one and two of the PTH signal, requires extracellular Ca^{2+} and is blocked by the Ca^{2+} channel blockers nicardipine and verapamil, but not diltiazem. By activating protein kinase C with 12-O-tetradecanoylphorbol 13-

acetate (TPA), they were able to mimic the rapid rise in $[Ca^{2+}]_i$ in phases one and two that PTH produced. These effects were also similar in time and concentration-dependence. They also found that stimulation of UMR 106 cells with PTH results in Ca^{2+} release from intracellular stores which appears to be mediated by a Ca^{2+}-dependent Ca^{2+} release mechanism: exposure of the cells to TPA resulted in partial depletion of the $[Ca^{2+}]_i$ stores, only when the extracellular solution contained Ca^{2+}. The close similarity between PKC-activation of Ca^{2+} channels and PTH-activation of Ca^{2+} channels in UMR-106 cells would suggest that phases one and two of the Ca^{2+} response are generated by activation of protein kinase C. Because phases one and two of the intracellular rise in Ca^{2+} require the presence of extracellular Ca^{2+} and are sensitive to Ca^{2+} channel blockers, and these responses were mimicked by PKC activation, this suggests two things: 1) the initial rise in Ca^{2+} is due to the opening of plasma membrane Ca^{2+} channels and 2) the activation of these channels is modulated by the protein kinase C pathway.

In addition, Yamaguchi *et al.* (1987a) were able to determine that stimulation of the slower phase three Ca^{2+} transient was due to an increase in cAMP. Addition of both forskolin, an adenylate cyclase activator, and 8-bromo cAMP, a membrane-permeable cAMP analogue, caused an increase in $[Ca^{2+}]_i$ that was dependent on extracellular Ca^{2+}, and was blocked by nifedipine and verapamil. This would suggest that phase three of the intracellular rise in Ca^{2+} reported by Yamaguchi *et al.* (1987a) is due to the activation of plasma membrane Ca^{2+} channels, and that these are probably activated by the adenylate cyclase pathway.

Although Yamaguchi *et al.* (1987 a,b) found a triphasic rise in $[Ca^{2+}]_i$ upon binding

of PTH to UMR 106 cells, and they attributed this to activation of the PLC and adenylate cyclase pathways, there remains much controversy about the precise intracellular mechanism of action of PTH on osteoblasts to increase $[Ca^{2+}]_i$. For instance, in a fura-2 study, Civitelli *et al.* (1992) found that in the presence of PTH, there was a rise in $[Ca^{2+}]_i$ in only 20% of the UMR 106 cells studied. Ljunggren *et al.* (1992) also found an inconsistent PTH-stimulated increase in $[Ca^{2+}]_i$, since only 11% of the UMR 106 cells studied responded with a rise in Ca^{2+}. The conflicting results regarding the effects of PTH on rises in $[Ca^{2+}]_i$ have been attributed to different stages of the cell cycle in UMR-106 cells (Wiltink *et al.*, 1993; Bizzarri *et al.*, 1994; Avioli *et al.*, 1995). For example, Bizzarri *et al.* (1994) have shown that UMR-106 cells respond to PTHrP and PTH preferentially when they are in the S phase of the cell cycle. In addition, the actions of PTH vary between different cell lines. For instance, Boland *et al.* (1986) found no significant rise in $[Ca^{2+}]_i$ upon PTH stimulation of G-292 cells, a human osteosarcoma cell line, ROS 25/1 and ROS 17/2.8, rat osteosarcoma cell lines, or in primary cultures of bone cells from neonatal mouse calvaria.

Since PTHrP and PTH have different potencies in different cell lines, and sometimes within the same cell line, one explanation for these differences could be a separate mechanism for signal transduction of PTHrP than for PTH. Civitelli *et al.* (1989) found that PTHrP caused a transient increase $[Ca^{2+}]_i$ in UMR 106 cells, with the shape and duration of the Ca^{2+} transient being very similar to that of the PTH-stimulated Ca^{2+} transient. However, the rise in $[Ca^{2+}]_i$ due to PTHrP still occurred after extracellular Ca^{2+} chelation, suggesting that the Ca^{2+} was released from intracellular stores following PLC

pathway activation. Yamaguchi *et al.* (1987 a,b) had found that PTH action was dependent upon extracellular Ca^{2+}, indicating the activation of plasma membrane Ca^{2+} channels, and therefore, a different means than PTHrP of increasing $[Ca^{2+}]_i$. Civitelli *et al.* (1989) also showed that the Ca^{2+} transient induced by PTHrP was unaffected by the Ca^{2+} channel blocker diltiazem, but was partially inhibited by the Ca^{2+} channel blocker nitrendipine; this suggests that there is some influx of Ca^{2+} through plasma membrane Ca^{2+} channels possibly activated by adenylate cyclase, but it is doubtful that this is the primary source of the intracellular rise in Ca^{2+}.

In summary, it appears that PTHrP and PTH regulate $[Ca^{2+}]_i$ in osteoblasts, and this may be an important factor in the process of bone resorption. However, the effects of PTHrP and PTH on increasing $[Ca^{2+}]_i$ in osteoblasts remains controversial. Not only is the increase in $[Ca^{2+}]_i$ inconsistent, but the intracellular mechanisms of action of PTHrP and PTH which cause the rise in $[Ca^{2+}]_i$ concentration remains unknown. One source of $[Ca^{2+}]_i$ entry is through voltage-dependent L-type Ca^{2+} channels in the plasma membrane. Since some of these channels are strongly regulated by second messenger pathways, it is important to further characterize the regulation of these channels by PTHrP and PTH.

1.6.3. Effects of PTHrP and PTH on the adenylate cyclase/ protein kinase (PKA) signal transduction pathway

The adenylate cyclase signal transduction pathway plays a major role in several cell systems for converting an extracellular signal into an intracellular response. Activation of adenylate cyclase by the appropriate ligand leads to the generation of cAMP which exerts

its physiological role through an interaction with protein kinase A (PKA). The targets of PKA are proteins that have specific structural or enzymatic properties, such as channel proteins; phosphorylation of these target proteins results in a significant change in their activity and/or conformation with specific physiological consequences. For instance, PKA, through its phosphorylation effect, may change the state of ion channels in membranes (Civitelli *et al.*, 1995).

For many years it has been thought that PTH acts via activation of the adenylate cyclase/cAMP pathway; this view was based on the fact that important affects of PTH (ie. increased bone resorption, production of hypercalcemia) could be mimicked by cAMP (Tregear et. al., 1973). However, it was shown that bone resorption could also be stimulated by increasing $[Ca^{2+}]_i$ in fetal rat bone cells (Dziak et. at., 1976). Furthermore, PTH fragments that failed to activate the adenylate cyclase pathway could still mimic some of the biological actions of PTH (Horiuchi *et al.*, 1983). Several investigators have confirmed that PTH, in addition to stimulating cAMP, also activates the Ca^{2+} second messenger system in a variety of cell models, including the UMR-106 cell line (Lowik *et al.*, 1985; Yamaguchi *et al.*, 1987).

Although PTHrP and PTH are thought to activate both the cAMP and Ca^{2+} message systems, the relative role that each pathway plays in the response to receptor activation remains unknown. There is extensive but conflicting evidence regarding the consistency of intracellular rises in Ca^{2+} after PTH receptor binding. There is a consistent increase in $[cAMP]_i$ concentrations following PTHrP or PTH application, but inconsistent PTHrP and PTH-stimulated increases in $[Ca^{2+}]_i$ (Ljunggren *et al.*, 1992; Civitelli *et al.*, 1994; Blind

et al., 1993). This would suggest that cAMP is the major second messenger activated following PTH receptor activation and that the PLC pathway may play a contributory role in bone resorption. In support of this hypothesis, Ljunngren *et al.* (1993) reported a significant inhibition of bone resorption induced by PTH in the presence of Rp-cAMPs, a cAMP analogue that competes with cAMP for binding sites on the regulatory subunit of PKA, in neonatal mouse calvarial cells. In contrast, Kaji *et al.* (1993) reported that Rp-cAMPs did not significantly antagonize PTHrP-stimulated bone resorption in mouse bone cells, indicating that, in addition to or rather than PKA activation, the stimulation of a second pathway, probably the PLC system, might play a major role in PTH signal transduction in these cells.

Studies have demonstrated "cross-talk" between the adenylate cyclase pathway and the phospholipase C pathways after PTH receptor binding. Recent evidence suggests that the cAMP pathway may be modulated by PKC in a time-dependent manner. For instance, Kitten *et al.* (1994) suggested that the activation of PKC first stimulates and then inhibits the action of adenylate cyclase in UMR-106 cells. Brief exposure (less than 1 hr) of UMR 106 cells to PMA, a PKC agonist, enhances PTH stimulation of adenylate cyclase, whereas more prolonged PMA treatment (more than 5 hrs) decreases the PTH response.

There is conflicting evidence regarding the potencies of PTHrP and PTH for increasing $[cAMP]_i$, once again suggesting that the two hormones stimulate different signal transduction pathways. For instance, Thorikay *et al.* (1989) found that PTHrP (1-141) was 5-fold more potent in stimulating cAMP synthesis in an osteosarcoma cell line than PTH (1-34), possibly reflecting a difference in signal transduction pathways between the two

hormones. In contrast, Blind *et al.* (1993) found that PTHrP (1-34) and PTH (1-34) had very similar effects on cAMP accumulation in UMR 106 cells, indicating that the two hormones have similar signal transduction pathways.

The role that cAMP plays in PTHrP and PTH signalling is significant, however, the precise extent and mechanism of this messenger's activation remain controversial. It is clear that further studies must be performed to elucidate the importance and exact physiological role of cAMP as a second messenger for these hormones.

1.6.4. Effects of PTHrP and PTH on the phospholipase C (PLC)/protein kinase C (PKC) signal transduction pathway

The PLC/PKC pathway plays a major role in cellular signalling, in addition to the cAMP mechanism, in many cell systems. Appropriate ligand binding activates Go proteins which subsequently stimulate PLC. When PLC is activated, it splits phosphoinositides in the cell membrane to inositol 1,4,5 triphosphate (IP_3), a potent stimulator of Ca^{2+} release from intracellular stores, and diacylglycerol (DAG), a PKC activator. Once PKC is activated, it phosphorylates target proteins in a similar manner to PKA. The phosphorylation of proteins then alters specific cellular actions, resulting in a cellular response (Civitelli *et al.*, 1995).

Controversy exists regarding the extent of PLC pathway activation by PTHrP and PTH in osteoblasts. It is well established that PTHrP and PTH consistently activate the adenylate cyclase pathway, however, there have been conflicting reports as to whether the PLC pathway is activated simultaneously in all cases, or if it is stimulated under selected

circumstances. Although it has been established that both the adenylate cyclase and PLC pathways are activated upon PTHrP/PTH receptor binding, it is unknown how these pathways interact to produce a cellular response.

It has been demonstrated that different domains of PTHrP and PTH stimulate different signalling pathways. For instance, it was found that the first two amino acids of PTHrP and PTH are essential to stimulate the cAMP pathway; however, if these two amino acids are absent, the two hormones will selectively activate the PKC pathway (van Leeuwen *et al.*, 1988). Fujimori *et al.* (1992) found that the 3-34 fragment of bovine PTH, which did not induce cAMP synthesis in UMR 106 cells, caused a transient increase in $[Ca^{2+}]_i$, indicating that while activation of the adenylate cyclase pathway requires amino acids 1 and 2 of bPTH, the PLC system requires a longer domain of the PTH N-terminus. In addition to the activation of PKC activity by PTH (3-34), reports found that bPTH (3-34) activates $[Ca^{2+}]_i$ release (Lowik *et al.*, 1985 and Fujimori *et al.*, 1992). Gagnon *et al.* (1993) found that the 28-34 amino acid domain of PTHrP is the same 28-34 domain of PTH that stimulates PKC activation, despite different primary amino acid sequences of the hormones. However, in addition to this 28-34 domain of PTHrP that stimulates PKC, Gagnon *et al.* (1993) found a second PKC activation domain, PTHrP (107-111) which caused maximal stimulation of PKC activity in ROS 17/2 cells. They suggested that this 107-111 fragment, instead of stimulating bone resorption as the 28-34 fragment does, inhibits bone resorption. This indicates possible multiple roles for PTHrP in both bone resorption and bone formation, depending upon which domain is activated.

As mentioned in the section 1.5.2., three phases of Ca^{2+} transients in UMR 106

cells have been described and attributed to different biochemical pathways (Yamaguchi *et al.*1987a,b). The two initial rapid transients induced by PTH were attributed to activation of PKC and subsequent PKC membrane translocation, when the opening of plasma membrane channels takes place. The third slower phase of the Ca^{2+} transient was explained by the activation of cAMP-dependent Ca^{2+} channels in the plasma membrane.

Tatrai *et al.* (1994) reported Ca^{2+} transients in UMR 106 cells in the presence of PTH. The transients were significantly smaller in the absence of extracellular Ca^{2+} than in its presence, although they were not abolished. This suggests that Ca^{2+} must be derived from the extracellular environment as well as from intracellular stores. This study partially supports that of Yamaguchi *et al.* (1987a,b) who concluded that an increase in $[Ca^{2+}]_i$ concentration was due to PKC activation of plasma membrane channels; however, in contrast Yamaguchi *et al.* (1987 a,b) attributed an increase in $[Ca^{2+}]_i$ due to a Ca^{2+}-dependent Ca^{2+} release mechanism of intracellular stores, which opposes Tatrai *et al.*'s finding that $[Ca^{2+}]_i$ release took place in the absence of extracellular Ca^{2+}. Tatrai and coworkers (1994) found that PTH-induced Ca^{2+} transients could be blocked by a PLC inhibitor, U-73122, providing supporting evidence for activation of the PLC pathway. It appears that the effect of PTH on inositol phosphate turnover is highly variable, since in some studies significant amounts of IP_3 accumulated after PTH stimulation (Civitelli *et al.*, 1988), whereas in other studies there was little or no IP_3 production (Tatrai *et al.*, 1994). Cell-cycle dependence of pathway activation may account for these variable observations.

In addition to the studies describing effects of PTHrP and PTH on Ca^{2+} transients and IP_3 activation, several investigators have demonstrated the effects of PTHrP and PTH

on protein kinase C (PKC) activity. For instance, Iida-Klein *et al.* (1989) showed that bPTH (1-34) increased cytosolic and membrane-bound PKC activity in UMR 106 cells by 12 and 157%, respectively, indicating that PTH stimulates the translocation of PKC from the cytosol to the membrane, where it probably targets Ca^{2+} channel proteins, accounting for the transient increase in $[Ca^{2+}]_i$ upon PTH receptor binding. Iida-Klein *et al.* (1989) also reported that forskolin, an adenylate cyclase agonist, failed to produce any significant effects on PKC activity. Variable results have been reported regarding PKC activation by PTHrP and PTH (Iida-Klein *et al.*, 1989; Bos *et al.*, 1994). This could be accounted for by the dependence of pathway activation on the stage of the cell cycle.

1.6.5. The interaction of the intracellular signalling pathways

In summary, it is well known that the adenylate cyclase and phospholipase C systems plays a significant role in PTHrP/PTH signalling; however, it is not known to what extent each of the pathways are involved or how these two pathways interact with one another to produce the cellular response. Traditionally, it has been assumed that PTHrP and PTH act through the same pathways to induce bone resorption, since studies have shown that the two hormones equipotently increase $[cAMP]_i$ (Blind *et al.*, 1993, Civitelli *et al.*, 1989). However, contradictory results have been reported on the ability of the two hormones to equipotently increase $[Ca^{2+}]_i$ (Civitelli *et al.*, 1992; Bizzarri *et al.*, 1994; Wiltink *et al.*, 1993). These differences in potencies between the two hormones suggests different intracellular mechanisms. When multiple signalling systems are activated an interaction between the intracellular pathways is often seen. It is evident that further studies

have to be performed to elucidate the signal transduction mechanisms of PTHrP and PTH and the possible interaction of adenylate cyclase and PLC pathways to produce a cellular response in osteoblasts.

1.7. Voltage-dependent calcium channels

In order for Ca^{2+} to enter a cell, it must pass through Ca^{2+} channels in the plasma membrane. There are several types of Ca^{2+} channels, including receptor-operated Ca^{2+} channels, second messenger-operated Ca^{2+} channels, stretch-activated Ca^{2+} channels and voltage-dependent Ca^{2+} channels (Duncan *et al.*, 1989). Since PTH receptor binding in bone cells usually involves changes in $[Ca^{2+}]_i$, Ca^{2+} channels in the membrane play a significant role in allowing an influx of Ca^{2+} into the cell and inducing a cellular response (Yamaguchi *et al.*, 1987). In the present study, electrophysiological methods are utilized; accordingly, it focuses primarily on voltage-dependent Ca^{2+} channels in UMR 106 cell membranes, a description of receptor-operated and second messenger-operated Ca^{2+} channels will not be included.

Voltage-dependent Ca^{2+} channels are a well-studied type of Ca^{2+} channel. Voltage-dependent Ca^{2+} channels, found in a wide variety of cells, have several features in common. For instance, voltage-dependent Ca^{2+} channels have a characteristic step depolarization-dependent activation (Fox *et al.*, 1987) (meaning that more channels open and remain open for longer periods of time as the membrane is depolarized to more positive potentials from a holding potential), and they bind Ca^{2+} with a high affinity (Tsien *et al.*, 1990). There are several subtypes of Ca^{2+} channels, including the L-type Ca^{2+}

channel, the T-type Ca^{2+} channel, the N-type Ca^{2+} channel, and the P-type Ca^{2+} channel. UMR 106 cells have L- and T-type Ca^{2+} channels, whereas the N-type Ca^{2+} channels occur only in neurons; the P-type Ca^{2+} channels are located in nervous tissue and their function has been attributed to the modulation of neurotransmitter release. T-type Ca^{2+} channels exist in UMR 106 cells (Li *et al.*, 1996), however, they are found mainly in the heart and neurons (Fox *et al.*, 1987). Since UMR 106 cells contain mainly L-type Ca^{2+} channels, this thesis focuses on this class of channels.

1.7.1. L-type calcium channels in bone

Voltage-dependent Ca^{2+} channels (VDCC) in bone cells have been studied for some time. For instance, fura-2 studies have indicated that Ca^{2+} influx through plasma membrane channels was mainly responsible for Ca^{2+} transients in several osteoblast cell lines (Yamaguchi *et al.*, 1987). To further support this notion, removal of extracellular Ca^{2+} or addition of voltage-dependent Ca^{2+} channel blockers, such as nifedipine and verapamil, inhibited the intracellular rise in Ca^{2+} observed in UMR-106 cells stimulated with PTH or PTHrP (Yamaguchi *et al.*, 1989, Civitelli *et al.*, 1989). These studies strongly suggest the presence of voltage-dependent Ca^{2+} channels in osteoblasts.

Although these studies suggested that VDCCs occur in bone cells, they did not provide direct evidence for the presence of these channels. Chesnoy-Marchais *et al.* (1988) were able to provide direct electrophysiological proof of the existence of L-type VDCC in newborn rat calvaria. By using the whole cell patch-clamp recording method, they were able to document both a slow inward Ca^{2+} channel current and a transient Ca^{2+} current.

Following their study, several other investigators were able to record similar L-type VDCC currents in other cell lines, such as UMR 106 and ROS 17/2.8 cells (Karpinski *et al.*, 1989; Yamaguchi *et al.*, 1989; Grygorczyk *et al.*, 1989; Duncan *et al.*, 1989; Li *et al.*, 1996). L-type Ca^{2+} channels are considered long-lasting channels since they inactivate slowly. The L-type Ca^{2+} channels are highly sensitive to dihydropyridines, and are activated when exposed to Bay K 8644 and inactivated in the presence of nifedipine (Karpinski *et al.*, 1989). In bone cells, L-type Ca^{2+} channels are activated between -30 and -20 mV and the peak inward currents exist between 0 and 40 mV. Single L-type Ca^{2+} channel conductance is between 21 and 28 pS when Ba^{2+} is used as the charge carrier (Tsien *et al.*, 1990).

1.7.2. Significance of studying L-type calcium channel modulation by PTHrP in bone

It is evident that the bone resorption process is extremely complex, with multiple pathways playing roles in Ca^{2+} mobilization. It has been suggested that the influx of Ca^{2+} through voltage-dependent Ca^{2+} channels in the plasma membrane, regulated by PTHrP and PTH, contributes to stimulating bone resorption. This is the first study to directly investigate the role of L-type Ca^{2+} channels in PTHrP and PTH signalling. Through the use of patch-clamping, the effects of PTHrP and PTH on L-type Ca^{2+} channels can be directly measured. In addition, the second messenger pathways which are activated by PTHrP and PTH and result in the regulation of L channels may be determined.

1.8. The specific objectives of this thesis

1. To demonstrate the effects of PTHrP on L-type Ca^{2+} channel currents in UMR 106 cells and to use the effects of PTH on the current as a means of comparison.

2. To investigate the second messenger mechanism(s) by which PTHrP modulates L-type Ca^{2+} channels in UMR 106 cells.

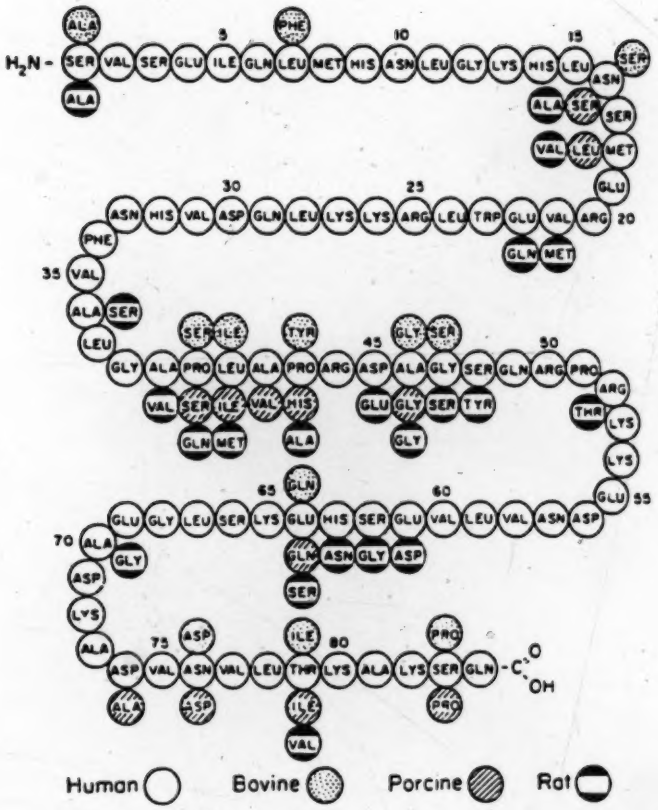

Fig. I-1. Schematic representation of the primary structure of mammalian PTH. The amino acid sequence of mammalian PTH (1-84) compares human with bovine, porcine, and rat hormone (from Coleman *et al.*, 1994).

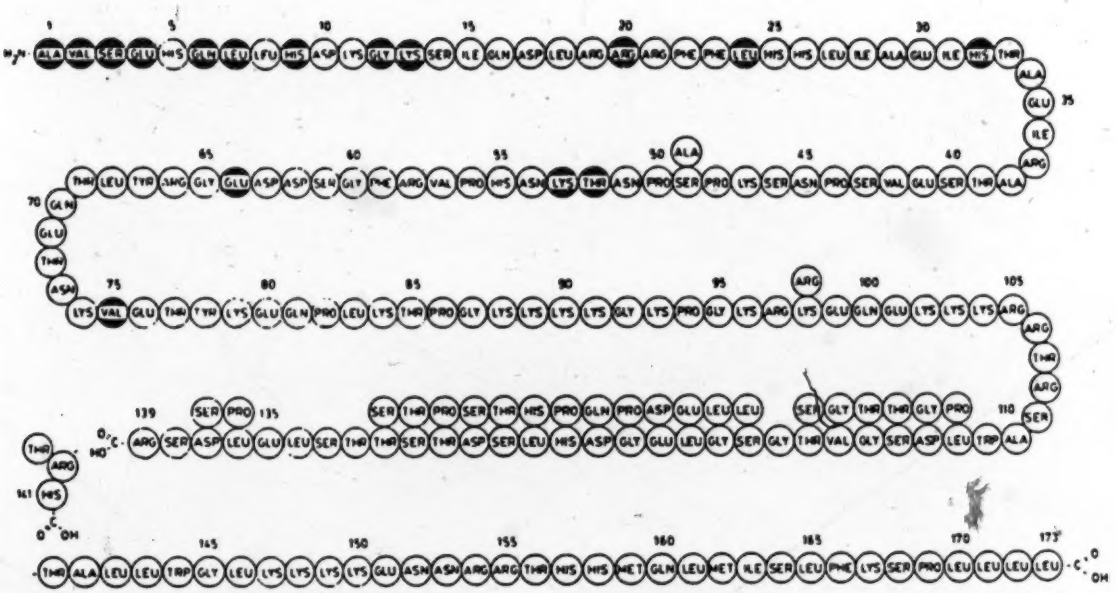

Fig. I-2. Schematic representation of the primary structure of human PTHrP. Closed circles illustrate the amino acids which are identical to PTH. In addition, the alternative C-terminus sequences of the human PTHrP isoforms of 139, 141, and 173 amino acids are shown (from Civitelli *et al.*, 1995).

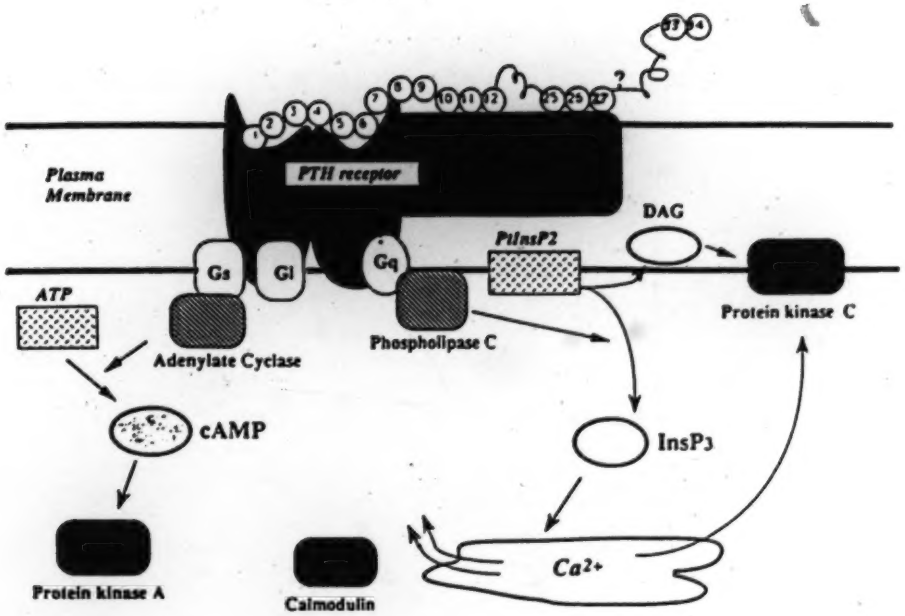

Fig. I-3. Schematic representation of PTH and/or PTHrP signal transduction. The first two amino acids of the PTH N-terminus are coupled to the adenylate cyclase pathway through stimulatory (G_s) and inhibitory (G_i) guanosine triphosphate-binding protein. Activation of adenylate cyclase leads to cAMP production and subsequent stimulation of protein kinase A, which dissociates into its regulatory and catalytic subunits. The phospholipase C pathway is stimulated by a domain of PTH beyond the first 3 amino acids, probably by a different G-protein, such as G_q. Once activated, phospholipase C catalyzes the hydrolysis of phosphatidylinositol 3,4-bisphosphate ($PtInsP_2$) into diacylglycerol (DAG) and $InsP_3$. DAG subsequently activates PKC, while $InsP_3$ mobilizes Ca^{2+} from intracellular storage pools. Increased $[Ca^{2+}]_i$ activates calmodulin and synergizes with protein kinase C (from Fujimori *et al.*, 1992).

Chapter II

Materials and Methods

2.1. Cell preparation

UMR 106 cells were used in this study. The UMR 106 cells were obtained from Dr. N. Partridge, St. Louis University, St. Louis, Missouri. The reasons for choosing UMR 106 cells for this study were as follows: 1) UMR 106 cells are a rat osteogenic sarcoma cell line originating from an osteoblastic sarcoma. Since this study focuses on the roles of PTHrP and PTH in bone resorption, these cells were ideal for these purposes. In addition, PTHrP/PTH receptors are widely distributed on UMR 106 cell membranes (Abou-Samra *et al.*, 1989; Okano *et al.*, 1994). 2) UMR 106 cells retain all of the characteristics of osteoblasts, including the presence of alkaline phosphatase activity and collagen synthesis, as well as cAMP accumulation in response to PTHrP and PTH. Therefore, the UMR 106 cell line is a good model to study the effects of PTHrP and PTH on bone.

UMR 106 cells (passages 3-30) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in room air. Dulbecco's Modified Eagle Medium (DMEM) (Gibco) containing 10% fetal bovine calf serum (FCS) (Gibco) was changed every 1-2 days. The cells usually became confluent after 5-6 days in culture. After 3 washes with Ca^{2+}-free Hank's buffered saline solution (HBSS) (Gibco), the cells were treated with 0.015% trypsin and 0.25 mM EDTA for 3 to 4 min. The harvested cells were then washed 2 times with DMEM

containing 10% FCS, and resuspended in the same medium for use in different experiments. For patch clamp studies, approximately $5x10^4$ cells were replated per 35 mm tissue culture dish in DMEM containing 10% FCS. The replated cells were used within 30 min. to 4 hours.

2.2. Patch clamp technique

2.2.1. Solutions

The compositions of the external (extracellular) solution and the internal (intracellular) solution were designed to separate the voltage-dependent Ca^{2+} channel currents from other ionic currents in whole cell experiments. The external solution contained (in mM): Tris, 110; $BaCl_2$, 20; CsCl, 5; HEPES, 20; KCl, 5; glucose, 20; TTX 500 nM. In all experiments, Ba^{2+} was used as the charge carrier instead of Ca^{2+} for the following reasons: 1) Ba^{2+} is more permeable than Ca^{2+} through L-type Ca^{2+} channels (Fox *et al.*, 1987; Tsien *et al.*, 1990). 2) Ba^{2+} in the external solution can block Ca^{2+}-activated K^+ channels. 3) Ba^{2+} also suppresses the potassium current through the delayed rectifier channel and the inward rectifier channel (Hagiwara *et al.*, 1978; Armstrong *et al.*, 1982). 4) Ba^{2+} prevents Ca^{2+}-induced Ca^{2+} channel inactivation (Armstrong *et al.*, 1980; Eaton *et al.*, 1980; Akbarali *et al.*, 1991). 5) The influx of Ba^{2+} during repeated depolarizations does not inhibit Ca^{2+} channels (Brown *et al.*, 1981; Lee *et al.*, 1985). The use of Tris in the external solution also reduces leakage and prolongs the viability of the cells (Moolenaar *et al.*, 1978). TTX was included in the external solution to block Na^{2+} channels.

The internal solution contained (in mM): Cs_2-aspartate, 70; EGTA, 5; $CaCl_2$ 0.5; ATP-Na_2, 2; $MgCl_2$, 5; K-pyruvate, 5; K-succinate, 5; glucose, 25; HEPES, 15; creatine phosphate-Na_2, 5; and creatine-kinase, 50 units/ml. The advantages of this composition are as follows: 1) Cs^+ ions block the K^+ channels from the inside of the membrane. 2) K^+-pyruvate and K^+-succinate support the citric acid cycle. 3) Creatine phosphate-Na_2, creatine kinase, and ATP facilitate the process of energy yield and utilization. Thus, contamination by ionic currents other than the Ca^{2+} current and the "run-down" of the Ca^{2+} channel current were minimized. As a result, in the present study, the Ca^{2+} current recorded could be maintained from at least 20 min to as long as 2 hours in some cells. Since 5 mM EGTA was included in the 0.5 mM Ca^{2+} internal solution, the concentration of intracellular free Ca^{2+} was estimated to be buffered at a concentration of less than $5x10^{8}$ M. Fura-2 was used to estimate the free Ca^{2+} concentration. The low concentration of $[Ca^{2+}]_i$, on one hand, helped to decrease the Ca^{2+}-induced inactivation of the Ca^{2+} current. On the otherhand, it decreased the activity of Ca^{2+}-dependent enzymes which, in turn, decrease or delay the effects mediated by these enzymes.

Before use, all solutions were filtered using a pore size of 0.22 μm in diameter. The osmolality of all solutions was adjusted to 320-340 mOsm and the pH was titrated to 7.4 using HCl, CsOH or Ba $(OH)_2$ as required.

For single channel measurements (cell-attached), the external solution contained the following (in mM): KCl 25; K^+-glutamate 120; HEPES-KOH 2; $MgCl_2$ 2; CaATP 2; EGTA 2; glucose 10. The pH was adjusted to 7.4 with NaOH. The high concentration of KCl in the external solution clamped the membrane potential at 0 mV. Pipettes were filled

with 70 mM $BaCl_2$/10mM Hepes (pH 7.4 with tetraethylammonium hydroxide)/110 sucrose. Bay K 8644 ($1x10^{-6}$M) was included in the pipette solution to promote L-channel opening. Without Bay K 8644 in the pipette, there were few or no channel openings.

2.2.2. Whole-cell and cell-attached recording

After the UMR 106 cells were washed 2 times with the external solution, the Petri dish with the cells was mounted onto the stage of an inverted phase contrast microscope (Nikon, Diaphot). The patch pipettes were fabricated from borosilicate (omega dot) thin-walled glass tubes (1.2 mm OD, 0.9 mm ID). They were pulled using a two stage microelectrode puller (Narishige MF-83). The resistance of the pipettes filled with the internal solution used for Ca^{2+} channel current recording was 2 to 5 megohms. The pipette solution was connected via an Ag/Cl electrode to an Axopatch-1B (Axon Instruments, Inc., CA) patch clamp amplifier. The standard gigaohm seal, whole-cell version of the patch clamp technique was used to measure whole-cell inward current (Hamill *et al.*, 1981). Before the pipette touched the cell membrane, the junction potential between the pipette and the bath solution was zeroed by adjusting the pipette current to zero. The pipette was placed onto the cell surface using a three-dimensional micromanipulator (Narishige). A tight seal (gigaseal) between the membrane and the tip of the pipette (10 to 50 gigaohm) was obtained by applying suction through the pipette. Further suction disrupted the membrane under the tip of the pipette and the pipette solution then dialysed the cell. Free access to the cytosol was reached when a capacitative current appeared and a membrane current could be recorded as a function of test potential.

The experiments were carried out at room temperature (20 to 22°C) in order to ensure a longer survival time of voltage-clamped cells and a better time resolution of the fast membrane currents. In the whole cell experiments, pulses of 200 msec duration were applied at intervals of 10 sec to allow complete recovery of Ca^{2+} channel inactivation. All signals were filtered at 1 KHz (4-pole low-pass Bessel filter), analog to digital converted and stored on the floppy disk of a personal computer (Zenith Data Systems) using pClamp software (version 5.7.1) and a DigiData 1200 interface (Axon Instruments).

In order to obtain a good space clamp, cells with a diameter of approximately 30 μm or less were chosen. (The diameters of UMR 106 cells were usually between 10 and 40 μm). The total cell capacitance ranged from 25 pF to 35 pF.

A universal phenomenon in patch-clamp studies is the decline of Ca^{2+} channel current with time ('run-down'). In UMR 106 cells, immediately after the cell was impaled, the inward current was usually small. However, the current increased gradually following 3 to 5 min and became relatively stable, as the result of the outward K^+ current being blocked by the high concentration of Cs^+ in the pipette and Ba^{2+} in the bath (Armstrong and Taylor, 1980; Quandt and Narahashi, 1984). If the current magnitude did not change from the third to the fifth minute after the rupture of the cell membrane, the rate of "run-down" of the inward current in UMR 106 cells was usually negligible for 30 min or longer. Therefore, only cells which had stable inward currents from the third to the fifth minute after penetration of the membrane were used. If the current decayed too quickly, it was difficult to distinguish "run-down" from the inhibitory or excitatory effect of the agents. Cells which showed a fast decline of inward current within this period were discarded.

Leakage currents were subtracted on line with the P/2 protocol using pClamp software. The subpulse used to subtract the leakage current did not activate any ionic current within the test pulse range examined. The product of series resistance and membrane current affect the accuracy of the voltage clamp. When there is a large series resistance, there is a deviation of the membrane potential from the command potential. In the present study, the peak currents were usually small (approximately 50 to 200 pA) with 20 mM Ba^{2+} as the charge carrier, and the series resistance was usually less than 10 megohms. The voltage error due to series resistance was less than 2 mV and, hence, series resistance compensation was not usually employed. In cases where the voltage error was greater than 2 mV, series resistance was compensated electronically using the Axopatch 1-B electronics.

The control I-V relationships were constructed using the peak values of the inward current (leakage corrected) and the currents were activated by progressive depolarization of the cell to various test potentials at a frequency of 0.5 Hz. Selected agents were then added directly into the bath solution and the change in the inward current was monitored continuously using test depolarizations at a frequency of 0.033 Hz. Since the agents required a period of 3 to 5 min to be distributed evenly throughout the bath solution, the I-V relationships representing the effects of the agents (ie. PTHrP) on Ca^{2+} current were usually obtained after at least a 3 min exposure of the cell to these test agents (ie. a PTHrP I-V relationship was obtained at 15 min). A 5% change (higher or lower) in the amplitude of the inward current was considered to be a significant change induced by the agents used in the experiments.

When performing single channel measurements, the cell-attached version was used. The holding potentials, duration of depolarization, and filter corner frequencies are indicated in the figure legends.

2.3. cAMP measurement

The cells were prepared as described above. Before the experiments, the culture medium was changed to DMEM with 0.1% BSA and the cells were equilibrated in this medium for 60 min. The cells were then incubated with selected agents in the same medium for 15 min. To measure intracellular cAMP, the drug containing DMEM was replaced by ice cold 5 mM acetic acid after 15 min. In order to release the [cAMP], the cells were lysed by repeated freezing and thawing twice in 5 mM acetic acid. The lysates were then transferred to centrifuge tubes and boiled for 5 min. The samples were stored at -20°C until analysis. The cAMP content was determined with a radioimmunoassay kit, using ^{125}I-cAMP as the label (Ho *et al.*, 1987).

2.4. Drugs

Bovine PTH (1-34) and bovine PTHrP (1-34) were obtained from Bachem Inc. (Torrance, CA) and dissolved in distilled water and stored in a -70°C freezer. Bay K 8644 (Calbiochem) and nifedipine (Sigma) were dissolved in DMSO. Dibutyryl guanosine 3'5'-cyclic monophosphate (db-cGMP) and 8-bromo adenosine cyclic monophosphate (8-bromo-cAMP) were purchased from Sigma and were dissolved in distilled H_2O. Forskolin was purchased from Sigma and was dissolved in DMSO. Rp-cAMPs was purchased from

Biolog Life Science Institute (FRG) and dissolved in distilled water. PMA was obtained from Sigma and dissolved in ethanol. The required concentration of the agent in the bath solution was achieved by adding the concentrated (1000x) stock solution to the dish. The final concentration of solvents had no detectable effect on the signals of interest in patch clamp. The other agents used in this thesis research were of the highest grades available from Sigma.

2.5. Statistics

Results are expressed as the means (plus, minus) the standard error of the mean (SEM). The peak inward current obtained from the current voltage relationship was used to determine the effect of the test agents. The paired Student's t-test or grouped t-test was used for comparison between mean values of the control and those obtained after drug administration. Analysis of variance in conjunction with the Newman-Keul's test was used for multiple group comparisons. P values less than 0.05 were considered statistically significant.

Single channel analysis (ie. all-points histogram) was carried out using the analysis programs in pClamp 6.05. Curve fits to the Hill and Boltzmann equations were performed using n-fit (ie. Levenberg Marquardt least squares algorithm). In the case of the Hill function, the highest concentrations used were taken as giving the maximum response. The Hill equation was of the form $B=\{B_{max}\}/\{1+(k/x)^n\}$ where k is the half maximal peptide concentration, x is the concentration of the peptide and n is cooperativity coefficient. The Boltzmann equation was of the form $V=\{V_{max}/[1+\exp(v_h-x)k]\}$ where v_h is the potential

at which half the channels are activated or inactivated, x is the voltage, and k is the slope factor.

Chapter III

Results - Part I:

Effects of PTHrP and PTH on the L-type calcium channel current in UMR 106 cells

3.1. Introduction

It has been shown that L-type Ca^{2+} channels are expressed in bone cells, such as rat neonatal calvaria (Chesnoy-Marchais, 1988), as well as ROS 17/2.8 and UMR 106 cells (Karpinski *et al.*, 1989). It has also been well documented that upon PTHrP and PTH binding to osteoblast-like cells (UMR 106), there is an increase in $[Ca^{2+}]_i$ (Yamaguchi *et al.*, 1987a,b; Civitelli *et al.*, 1989). Since this increase in $[Ca^{2+}]_i$ is, in part, dependent upon the presence of extracellular Ca^{2+} and can be inhibited by Ca^{2+} channel blockers such as verapamil (Yamaguchi *et al.*, 1987a,b; Civitelli *et al.*, 1989), it is very likely that the increase in $[Ca^{2+}]_i$ occurs through Ca^{2+} channels in the plasma membrane. It has also been proposed that the way in which osteoblasts signal osteoclasts to induce bone resorption after PTHrP and PTH binding is stimulated by this increase in $[Ca^{2+}]_i$. Therefore in order to investigate the possibility that PTHrP and PTH modulate this cellular response through the regulation of the L-type Ca^{2+} channels in UMR 106 cells, this chapter of the study has four main focuses:

1. To characterize the whole cell L-type channel current in UMR 106 cells using dihydropyridines, Ca^{2+} channel agonists and Ca^{2+} channel antagonists.

2. To characterize the single L-type channel in UMR 106 cells.

3. To investigate the effects of PTHrP on the single L-type Ca^{2+} channel current in UMR 106 cells.

4. To demonstrate the effects and concentration-dependence of PTHrP and PTH on L-type Ca^{2+} channels in UMR 106 cells.

3.2. Experimental design

The whole cell version of the patch clamp technique was used in these experiments to characterize the effects of dihydropyridines (Bay K 8644 and nifedipine) on the L-type Ca^{2+} channel current as well as to study the effects of PTHrP and PTH on the L-type Ca^{2+} channel current in UMR 106 cells. In addition, the cell- attached version of the patch clamp technique was used to measure the effects of PTHrP on the single L-type Ca^{2+} channel currents.

To investigate the effects of PTHrP and PTH on the L-type Ca^{2+} channel current in UMR 106 cells, the inward current was measured before and after the addition of PTHrP and PTH. A current-voltage (I-V) relationship was also obtained before and after the addition of PTHrP and PTH.

3.3. Results

3.3.1. Inward calcium currents (whole cell)

The membrane was held at -40 mV and step-depolarizations from -30 mV to +50 mV in 10 mV increments were applied which activated an inward current. At a holding

potential of -40 mV, T-channel currents, which are sometimes present in these cells, (Li *et al.*, 1996) were inactivated. The large outward K^+ currents were blocked by Cs^+ in the pipette solution and Ba^{2+} in the bath solution.

3.3.2. The L-type calcium channel current

In order to verify that the current being measured was the L-type Ca^{2+} channel current, dihydropyridines (DHP's) were used, since it is known that L-type channels are highly sensitive to DHP's. Fig. III-1 shows the I-V relationship and current record obtained at a test pulse of +20 mV before and after the addition of Bay K 8644 and nifedipine in a single UMR 106 cell. At a concentration of $3x10^{-6}$M, Bay K 8644 increased the current peak amplitude from -75 pA to -260 pA; at a concentration of $3x10^{-5}$M, nifedipine decreased the current peak amplitude from -260 pA to -10 pA. The peak of the I-V relationship was shifted towards more negative potentials. This is characteristic of the actions of Bay K 8644, since it changes the gating of the L- channel so that the mean open time of the channel increases (Tsien *et al.*, 1986). Fig. III-2 illustrates that at a concentration of $3x10^{-6}$M, Bay K 8644 increased the peak current amplitude by 194.0 ± 27.0 % (n=6); at a concentration of $3x10^{-5}$M, nifedipine, in the presence of Bay K 8644, decreased the peak amplitude of the current by 93.5 ± 0.75% (n=5).

3.3.3. The single L-type calcium channel current in UMR 106 cells

In Fig. III-3A,B, and C, the single L-type Ca^{2+} channel current is characterized. Fig. III-3A shows a typical cell-attached single L channel current obtained from a UMR

106 cell. The membrane was held at -50 mV and depolarized to +10 mV. A downward deflection represents the open state of the channel. The experiments were performed in low light conditions since $1x10^{-6}$M Bay K 8644 (a light-sensitive dihydropyridine) was included in the pipette solution to promote the opening of channels. Fig. III-3B demonstrates an all-points current amplitude histogram, which represents the current amplitudes that are generated when the cell membrane is depolarized to +10 mV in a single UMR 106 cell. The all-points amplitude histogram was separated into two Gaussian components. One large peak was produced at 0 pA, which corresponded to the large number of baseline sample points, while a smaller second peak was generated at approximately -0.85 pA, which corresponds to the relatively fewer number of single channel open points. This suggests that when the cell is depolarized to +10 mV, the amplitude of the current that is produced when the channels open is approximately -0.85 pA. Fig. III-3C illustrates an I-V relationship for single L-type Ca^{2+} channel currents in UMR 106 cells. When the membrane was held at -50 mV and depolarized to 10 mV, 0 mV, and +10 mV, the average current amplitudes that were generated were -1.30 (n=1), -1.06 (n=4), and -0.85 pA (n=4), respectively. When the cell was depolarized to -10 mV, there were very few channel openings and only one cell was obtained for this potential. The estimated conductance of this channel is 25 pS.

3.3.4. Run-down of the calcium channel current in UMR 106 cells

In *in vitro* situations L-type Ca^{2+} channel currents run down in cells. Many factors contribute to the run-down of currents in cells. For example, intracellular consituents, such

as ATP, are required to maintain the Ca^{2+} channel activity, and may become diluted due to the dialysis with the pipette solution. The rate of run-down can vary from cell to cell, or in different cell lines. In the UMR 106 cells used in the experiments, the current was initially outward, but then quickly became inward as the Cs^{+} in the pipette solution dialysed the cell and blocked the K^{+} channels. The maximum inward Ca^{2+} current was reached once the Cs^{+} completely blocked the K^{+} channels, which was usually after a time period of 3- 5 min. After the current reached a maximum, this time was taken as 0 min. In Fig. III-4, currents were leakage corrected on-line and the peak inward current was plotted at 2 min. intervals (n=4). All current values were normalized to the peak inward current at 0 min. In the majority of cells, the current amplitude remained at the same level for 30 min. or longer. The fact that the current did not run down at a faster rate could be due to several factors: 1) A very low concentration of trypsin (0.015%) was utilized to remove the cells from the culture dishes, which may have minimized the damage to the plasma membranes of the UMR 106 cells. 2) Cs^{+}-aspartate and EGTA were included in the pipette solution and function as strong Ca^{2+} and Ba^{2+} chelators. This keeps the levels of Ca^{2+} and Ba^{2+} inside the cell low which would aid in decreasing the run-down of the cell. 3) ATP, K^{+}-succinate, K^{+}-pyruvate, and creatine kinase were also included in the pipette solution and provided the cell with an energy source which may have helped to reduce the run-down of the Ca^{2+} channel currents.

Since the run-down of the L-type Ca^{2+} channel current in UMR 106 cells was very slow and quite small, it could be concluded that any decrease in the current after addition of drugs is likely due to the effect of the drug on the L-type Ca^{2+} channel current, and not

due to the run-down of the Ca^{2+} channel current.

3.3.5. Effect of PTHrP on the L-type calcium channel current

To investigate the effect of PTHrP on the L-type channel in a UMR 106 cell, an I-V relationship was obtained before and after the addition of PTHrP to the bath solution. A current record, at +20 mV, and the corresponding I-V relationship are shown in Fig. III-5. After the current reached a steady state and the control current was obtained, a single concentration of PTHrP was added to the external solution. The maximum increase in the inward Ca^{2+} current occurred at approximately 15 min. At a concentration of $6.25x10^{-8}$M, PTHrP increased the L-type current from -80 pA to -230 pA.

Not every cell responded to PTHrP. The rate of response was approximately 30%. The data shown in this thesis represents only those cells that responded to PTHrP.

3.3.6. Time-course of the effect of PTHrP on the L-type calcium channel current

The effect of PTHrP on the L-type Ca^{2+} channel current was measured as a function of time. In Fig. III-6, currents were leakage-corrected on-line and the peak inward current was plotted at 1 min. intervals (n=3) . All current values were normalized to the peak inward current at 0 min (see Fig. III-4). At 0 min., PTHrP was added to the external solution. The maximal effect of PTHrP occurred at approximately 14 min. Since the drugs were added to the external bath by an HPLC injector at a distance of approximately 8 mm, some time was needed for the drug to diffuse to the cell. Therefore, the 14 min. period of

time for the Ca^{2+} channel currents to increase upon addition of PTHrP includes both the diffusion time and the time in which PTHrP generates second messengers inside the cell to cause an increased influx of Ca^{2+} through the L-type Ca^{2+} channels in the membrane.

3.3.7. Effect of PTH on the L-type calcium channel current

To investigate the effect of PTH on the L-type channel in a UMR 106 cell, an I-V relationship was obtained before and after the addition of PTH to the bath solution. The I-V relationship and the current, at a test potential of +20 mV, are shown in Fig. III-7. After the current reached a steady state and the control current was obtained, a single concentration of PTH was added to the external solution. The maximum increase in the Ca^{2+} current occurred at approximately 15 min after the addition of PTH. At a concentration of $1x10^{-8}$M, PTH increased the L-type current from -180 pA to -345 pA.

Not every cell responded to PTH. The rate of response was similar to cells described in section 3.3.5.

3.3.8. Effect of PTHrP and PTH on the L-type calcium channel current in UMR 106 cells as a function of concentration

To determine if PTHrP and PTH have the same potencies on the L-type Ca^{2+} channel current in UMR 106 cells, the effect of the two peptides as a function of concentration was measured. The effect of each concentration of PTHrP that was given was measured in a separate cell, therefore the administration of concentrations was not cumulative. Fig. III-8A demonstrates the effects of five concentrations of PTHrP on the

L-type Ca^{2+} channel current. The cell membrane was held at -40 mV and the peak inward current was measured before and after addition of PTHrP. At 15 min., an I-V relationship was generated and the change in the peak inward current was taken as the maximum increase. The increase in the current after the addition of PTHrP was normalized to the control (see Fig. III-4). PTHrP produced a maximal increase of 122.25±25.35% at a concentration of $6.25x10^{-8}$M PTHrP. When concentrations of $1x10^{-7}$M PTHrP or greater were added to the cells, the current decreased significantly (data not shown). This suggests that this concentration is either too toxic to the cells or it may be activating a different pathway than normal.

Fig. III-8B demonstrates the effects of five concentrations of PTH on the L-type Ca^{2+} channel current. The cell membrane was held at -40 mV and the peak inward current was measured after the dose of PTH was administered. The current reached a maximum at approximately 15 min. after the addition of PTH and an I-V relationship was measured at this time (see section 3.3.7.). The increase in the peak current after the addition of PTH was normalized to the control (see Fig. III-4). PTH produced a maximal increase of 99.2±24.3 % at a concentration of $7.5x10^{-6}$M.

3.3.9. Effect of PTHrP on the steady-state activation and inactivation of the L-type calcium channel current in UMR 106 cells

In Fig. III-9A, the steady-state activation as described by the normalized conductance of the L-type Ca^{2+} channel current is illustrated. The magnitudes of the normalized conductance were measured from tail currents. A 500 μsec segment was

blanked to remove imperfect transient cancellation. The control current was activated at a membrane potential between -30 mV to -20 mV and the peak inward current occurred at +20 mV. After the addition of 6.25×10^{-8}M PTHrP, the potential at which the current was activated remained between -30 mV and -20 mV and the peak inward current also remained at +20 mV. Because there was no significant shift in the potential at which the L-type current was activated after the addition of PTHrP, this would suggest that PTHrP does not have a significant effect on the steady-state activation of the L-type Ca^{2+} channel in UMR 106 cells.

In Fig. III-9B, the steady-state inactivation as described by the normalized conductance of the L-type Ca^{2+} channel current is illustrated. The magnitudes of the normalized conductance were measured from tail currents. A 500 μsec segment was blanked to remove imperfect transient cancellation. The control current started to inactivate at a membrane potential between -40 mV to -30 mV and the current was completely inactivated at +10 mV. After the addition of 6.25×10^{-8}M PTHrP, the potential at which the current was inactivated remained between -40 mV and -30 mV and the peak current inactivation also remained at +10 mV. Because there was no significant shift in the potential at which the L-type current was inactivated after the addition of PTHrP, this would suggest that PTHrP does not significantly affect the steady-state inactivation of the L-type Ca^{2+} channel in UMR 106 cells.

3.3.10 Effect of PTHrP on single L-type calcium channels in UMR 106 cells

Fig. III-10A illustrates single channel recordings in UMR 106 cells. All of the

traces were obtained from a single cell-attached patch. A seal was obtained and the cell was left to stabilize for 5-10 min before control currents were recorded. Bay K 8644 ($1x10^{-6}$M) was included in the pipette solution to promote the opening of the Ca^{2+} channels. This experiment was performed under low light conditions since Bay K 8644 inactivates when exposed to light. The membrane patch was held at -50 mV and depolarized to +10 mV. A downward deflection represents the open state of the channel. The amplitude of the L-type Ca^{2+} channel current at this test potential was approximately -0.85 pA (see Fig. III-3B,C). Fig. III-10B shows the effect of $1x10^{-9}$M PTHrP on the single L-type Ca^{2+} channels in UMR 106 cells. The effect of PTHrP on the Ca^{2+} channel current was measured approximately 15 min. after the addition of PTHrP. After the addition of PTHrP, by observing single L-type Ca^{2+} channel recordings, the number of channel openings appeared to increase and the channel open time also appeared to increase.

3.3.11 The effect of PTHrP on the open time distribution of the single L-type calcium channel current in UMR 106 cells

In order to confirm that PTHrP increases the open time of the L-type Ca^{2+} channels in UMR 106 cells, an open time distribution was obtained before and after the addition of PTHrP. The open time distributions were obtained from 32 separate 250 ms records in a single cell experiment. Fig. III-11A shows the open time distribution of a single L-type Ca^{2+} channel when the membrane was held at -50 mV and depolarized to +10 mV (see Fig. III-10A). The time constant obtained by curve fitting the distribution to a single exponential was calculated to be τ = 0.92 ms. Fig. III-11B illustrates the open time

distribution of the L-type channel after the addition of $1x10^{-9}$M PTHrP. The time constant increased to τ = 1.56 ms. This suggests that PTHrP increases the mean open time of the L-type Ca^{2+} channels in UMR 106 cells. In addition, a P(open) analysis was performed which provides a relative measure of the time that a channel spends in the open state. This is calculated by: $P(open)=[t_o/t_i]/N$, where t_o is the total open time for the level under consideration, t_i is the time interval over which P(open) is measured, and N is the number of channels in the patch. Before the addition of PTHrP, P(open) was calculated to be approximately 0.007; after the addition of PTHrP, P(open) was calculated to be approximately 0.038. This further supports the observation that PTHrP increases the time in which the channel remains open.

3.4. Discussion

This chapter focused on the effects of PTHrP and PTH on the L-type Ca^{2+} channel current in UMR 106 cells. The L-type Ca^{2+} channel was first characterized, and it was verified that the channels being studied were L-type Ca^{2+} channels since they were highly sensitive to the dihydropyridines Bay K 8644 and nifedipine. Single channel measurements were also performed, and it was determined that the average current amplitudes at -10 mV, 0 mV, and +10 mV were approximately -1.30 pA, -1.06 pA, and -0.85 pA, respectively. The estimated conductance of this channel was 25 pS when Ba^{2+} was used as the charge carrier.

A further experiment involved obtaining a current-voltage (I-V) relationship before and after the addition of PTHrP and PTH in two separate UMR 106 cells. An I-V

relationship is an important tool to characterize ion channels. The concept behind obtaining an I-V is to apply brief test pulses of different amplitudes from a holding potential. The measured current-voltage relationship represents the result that the membrane potential has on the activation of specific ion channels, in addition to the force that drives ions through the channel. This is determined by both the membrane potential and the difference in ion concentration across the membrane. Thus, for a given type of channel, the I-V relationship depends on the characteristics of the channel itself, and on the concentration of the permeant ion. It was found that PTHrP, at a concentration of 6.25×10^{-8}M, increased the current amplitude from -80 pA to -230 pA. In addition to PTHrP, the effect of PTH at a concentration of 1×10^{-8}M was obtained. At this concentration, PTH increased the Ca^{2+} current from -180 pA to -345 pA.

The effects of PTHrP and PTH on the L-type Ca^{2+} current as a function of concentration were also investigated. It was found that PTHrP was significantly more potent in increasing the L-type Ca^{2+} current than PTH. In addition, the slope of the PTHrP curve was higher than the slope of the PTH curve. The differences in the potencies and slopes of these hormones could be due to the following: 1) PTHrP may have a higher affinity than PTH for the classical PTHrP/PTH receptor in UMR 106 cells 2) In addition to the classical PTHrP/PTH receptor that exists in UMR 106 cells, there may be a second type of receptor, both of which may operate through different second messenger pathways 3) PTHrP and PTH may activate different signal transduction pathways upon binding to the classical PTHrP/PTH receptor. Chapter IV investigates the second messenger pathway(s) through which PTHrP operates.

Finally, the effect of PTHrP on the steady-state activation and inactivation of the L-type Ca^{2+} channel current in UMR 106 cells was investigated. If the steady state activation or inactivation were to shift to more negative potentials, this would suggest that PTHrP modulates the gating of the channel. However, it was found that PTHrP did not significantly shift the steady state activation or inactivation.

The effect of PTHrP on single L-type Ca^{2+} channel currents was also investigated. It was found that PTHrP increased the time constant which was fitted to the open time distribution (ie. before PTHrP, τ = 0.92 ms; after PTHrP, τ = 1.56 ms). In addition, when the relative time of the channel in the open state (P_o) was calculated, it increased from 0.007 to 0.038 after the addition of PTHrP. The single channel recordings before and after the addition of PTHrP, suggested that the number of openings also appeared to increase. This suggests that PTHrP does affect the gating of the channel by increasing the open time of the channel in addition to increasing the probability of channel opening. It should be noted, however, that Bay K 8644, which modulates the gating of L-type Ca^{2+} channels, was present to promote the opening of L-type channels. Therefore, the effect of PTHrP on single L-type channels was studied under nonphysiological conditions. However, since Bay K 8644 degrades, even under low-light conditions, the concentration of Bay K 8644 present when the effect of PTHrP was being measured was likely much lower than it was when the control current records were obtained. As a result, the effect of PTHrP on the channel is likely to be a genuine effect, since levels of Bay K 8644 by this time would be lower than when the control currents were obtained.

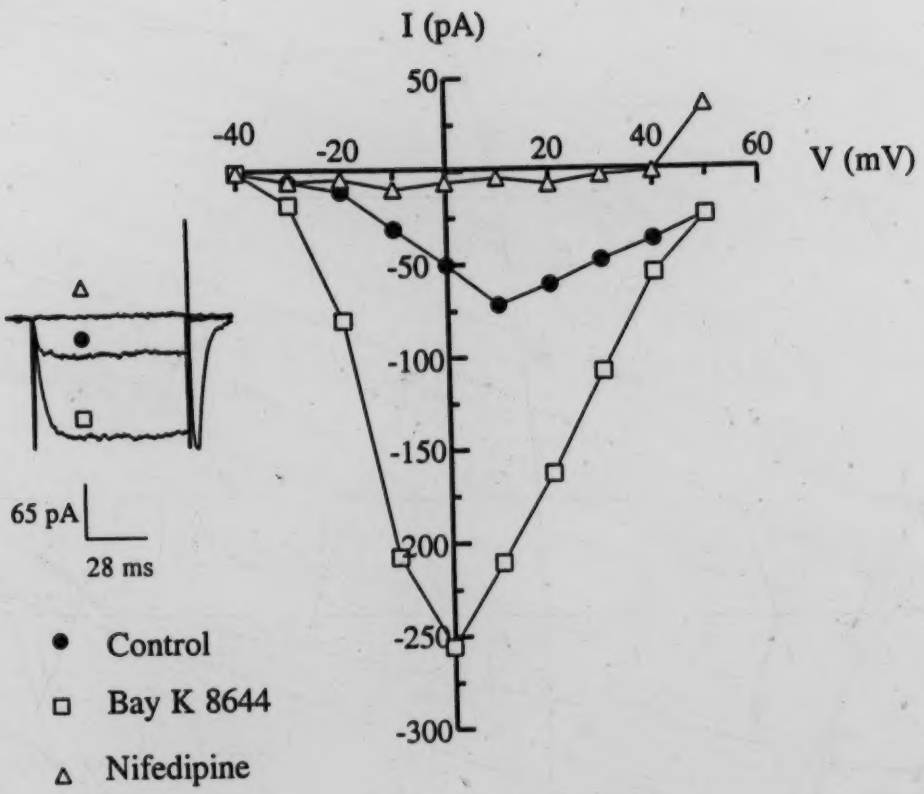

Fig. III-1. The effects of Bay K 8644 and nifedipine on the L-type Ca^{2+} channel current in a UMR 106 cell. The current records shown at the left were activated by depolarizing the cell to +10 mV from a holding potential of -40 mV. The membrane was held at -40 mV to assure that T-channel currents were not activated (Li *et al.*, 1996). The control current (closed circles), the current 5 min. after the application of $3x10^{-6}$M Bay K 8644 (open circles), and the current 5 min. after the application of $3x10^{-5}$M nifedipine (triangles) are shown. The I-V relationship before and after the addition of Bay K 8644 and nifedipine are shown at the right. Bay K 8644 increased the peak amplitude from -75pA to -260pA and shifted the peak of the I-V relationship from +10 mV to 0 mV. Nifedipine, in the presence of Bay K 8644, decreased the peak current amplitude from -260 pA to -10 pA.

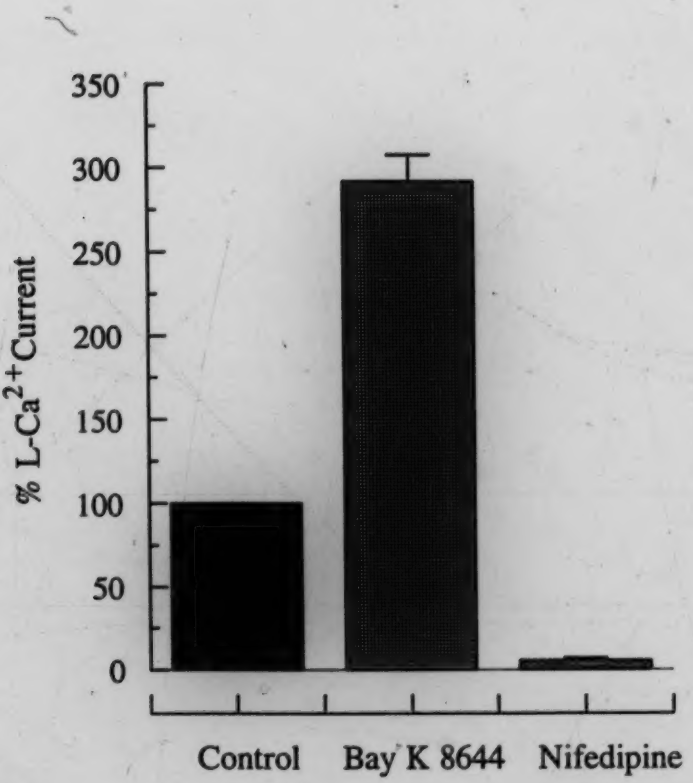

Fig. III-2. The effects of Bay K 8644 and nifedipine on the L type Ca^{2+} channel in a group of UMR 106 cells. At a concentration of $3x10^{-6}$M, Bay K 8644 increased the peak amplitude of the L-type Ca^{2+} current to 194 ± 27% ($\bar{x}$ ± S.E.M., n=6) of the control level (100%); nifedipine, at a concentration of $3x10^{-5}$M, decreased the peak amplitude of the L-type calcium current to 6.5 ± 0.75% ($\bar{x}$ ± S.E.M., n=5) of the control level (100%).

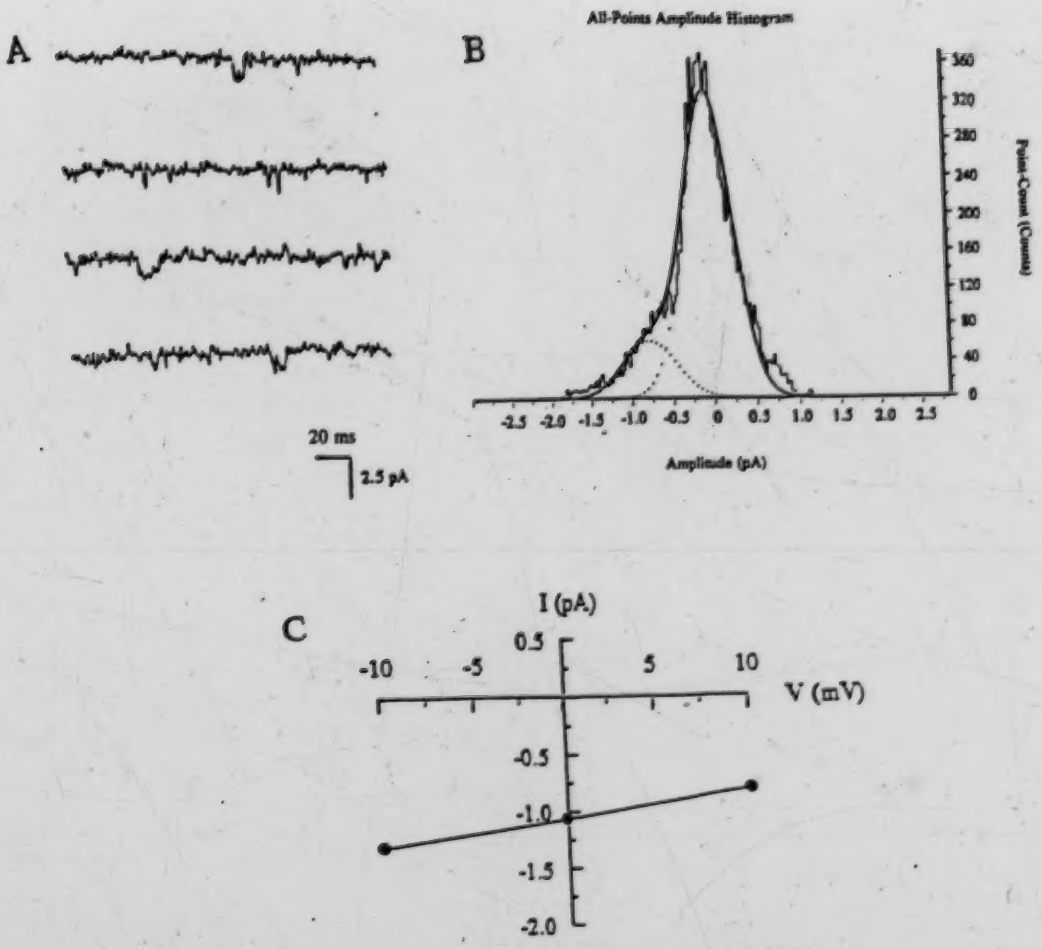

Fig. III-3 A. Current recordings of a single L-type Ca^{2+} channel in a cell-attached patch. The membrane was held at -50 mV and depolarized to +10 mV. A downward deflection represents an open state of the channel. $1x10^{-6}$M Bay K 8644 was included in the pipette solution to promote the opening of the channel. The current was filtered at 300 Hz.

B. An all-points current amplitude histogram showing an open state of the L-type Ca^{2+} channel at approximately -0.85 pA in a single UMR 106 cell. This histogram represents the amplitude of the L-type calcium current when the membrane is held at -50 mV and depolarized to +10 mV. The dotted lines show the separation of the two Gaussian components.

C. I-V relationship of the single L-type Ca^{2+} channel in a group of UMR 106 cells. The membrane was held at -50 mV and depolarized to -10 mV, 0 mV, and +10 mV. Few channel openings were seen at -10 mV and recordings at this potential were obtained from one cell. At the potentials of 0 mV and +10 mV, recordings were obtained from 4 cells and the current magnitudes were obtained as shown in Fig. III-B. At -10 mV, the average current amplitude was -1.3 pA. At 0 mV, the average current amplitude was -1.06 pA and at +10 mV, the average current amplitude was -0.85 pA. The conductance was estimated to be 25 pS.

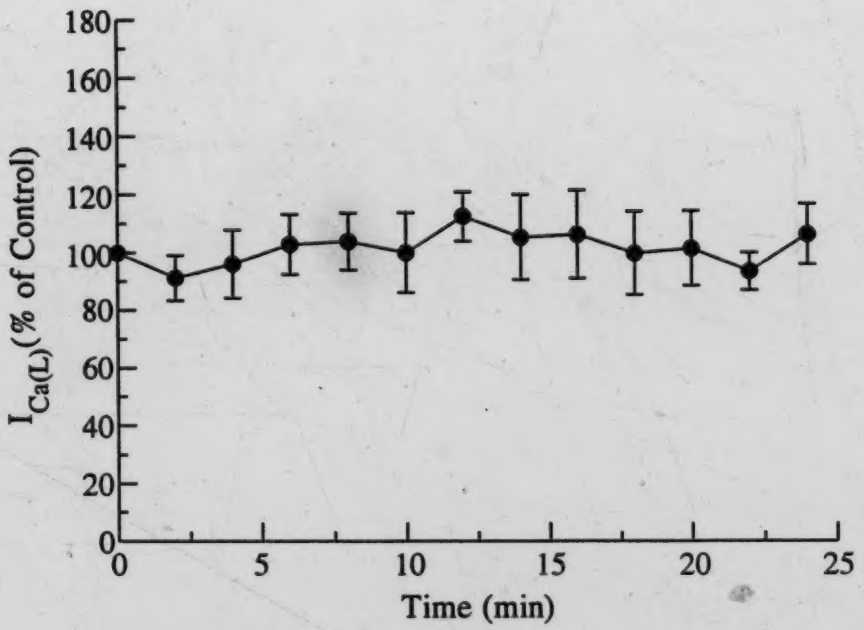

Fig. III-4. Run-down of the L-type Ca^{2+} channel current in UMR 106 cells. Currents were leakage corrected on-line and the peak inward current was plotted at 2 min. intervals ($\bar{x} \pm$ S.E.M., n=4). All current values were normalized to the peak inward current at 0 min. 0 min. was defined as the point at which the current became stable (3-5 min) (ie. Cs^+ blocked the outward K^+ current and the internal dialysis was complete).

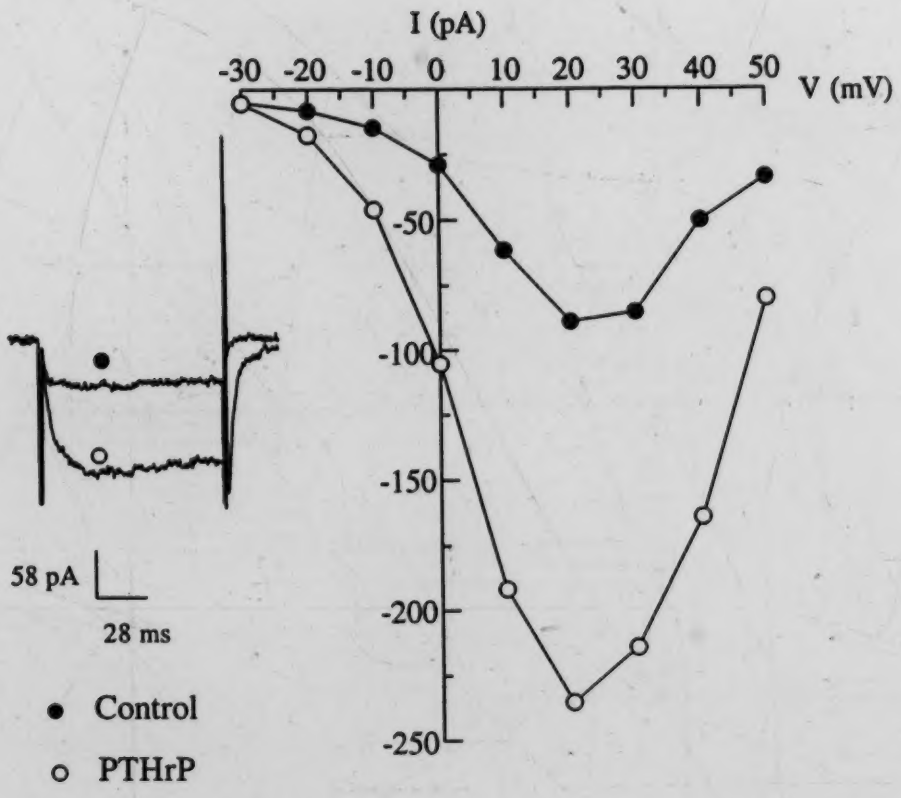

Fig. III-5. Peak inward current (leakage corrected) and the corresponding I-V relationship recorded from a UMR 106 cell before and after the addition of 6.25×10^{-8}M PTHrP. The peak L-channel current was activated by depolarizing the cell from a holding potential of -40 mV to +20 mV. 15 min. after the addition of PTHrP, the current increased from -80 pA to -230 pA.

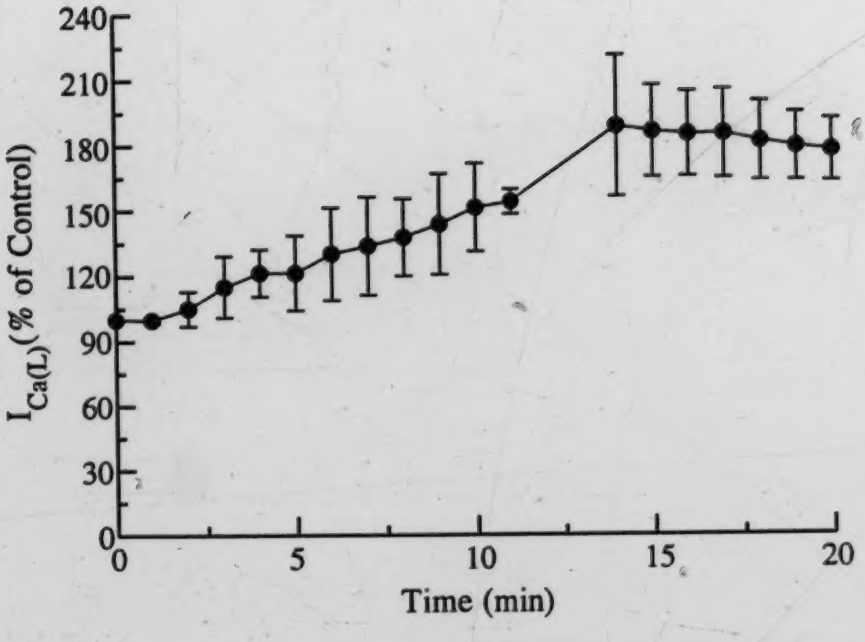

Fig. III-6. The effect of PTHrP on the L-type Ca^{2+} channel current in UMR 106 cells as a function of time. Currents were leakage corrected on-line and the peak inward current was plotted at 1 min. intervals ($\bar{x} \pm$ S.E.M., n=3). All current values were normalized to the peak inward current at 0 min. 0 min. was defined as the point at which the current became stable due to internal dialysis being complete, and PTHrP was added to the external solution. The maximal effect of PTHrP occurred at approximately 14 min.

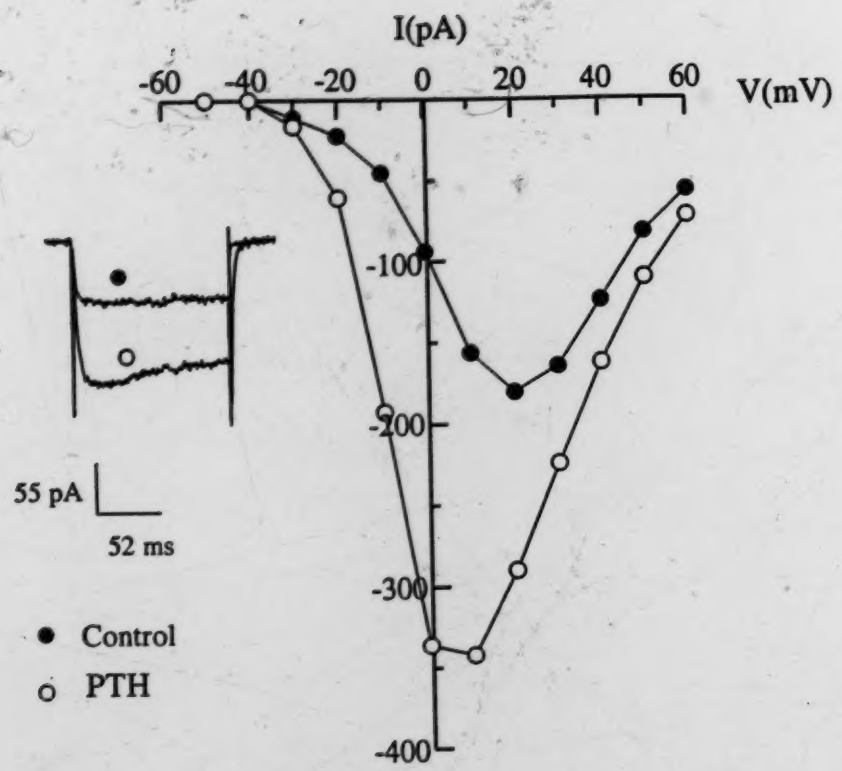

Fig. III-7. Peak inward current (leakage corrected) and the corresponding I-V relationship recorded from a UMR 106 cell before and after the addition of $1x10^{-8}$M PTH. The control peak L-channel current was activated by depolarizing the cell from a holding potential of -40 mV to +20 mV. 15 min. after the addition of PTH, the current increased from -180 pA to -345 pA.

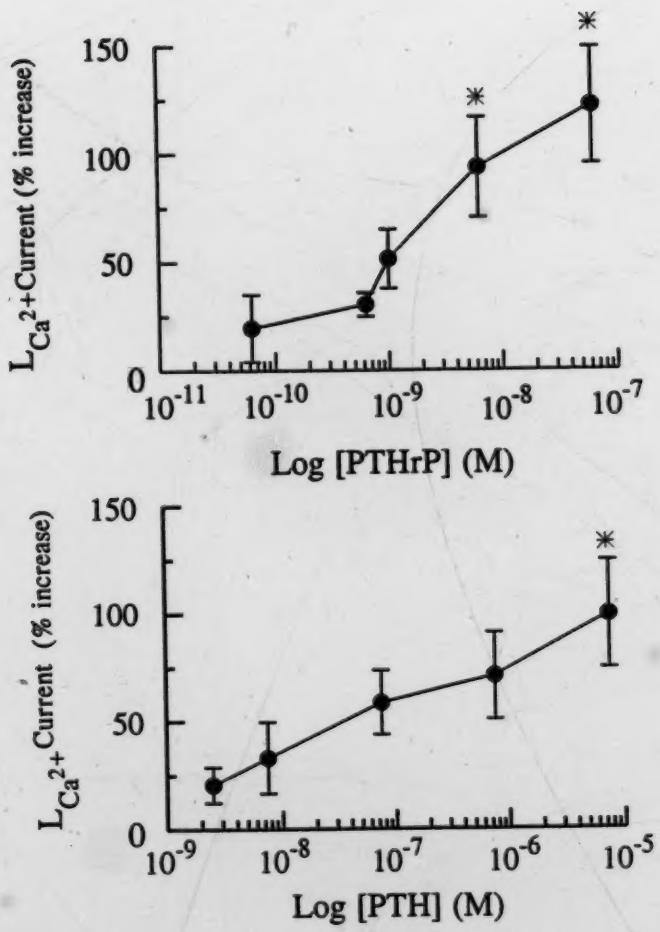

Fig. III-8 A. The effect of five concentrations of PTHrP on the L-type Ca^{2+} channel current recorded in UMR 106 cells. The current obtained after a 15 min. exposure to $6.25x10^{-11}$M (n=5), $6.25x10^{-10}$M (n=5), $1.0x10^{-9}$M (n=2), $6.25x10^{-9}$M (n=8), and $6.25x10^{-8}$M (n=11) PTHrP was normalized to the control. PTHrP produced a maximal increase of 122.25 ± 27.04 % at $6.25x10^{-8}$M. (*) The % increase of the L-Ca^{2+} current is significantly different than at the concentrations of $1x10^{-9}$M, $6.25x10^{-10}$M and $6.25x10^{-11}$M PTHrP. ($p<0.05$ - Newman-Keul's).

B. The effect of five concentrations of PTH on the L-type Ca^{2+} channel current in UMR 106 cells. The current obtained after 15 min. exposure to $2.5x10^{-9}$M (n=2), $7.5x10^{-9}$M (n=5), $7.5x10^{-8}$M (n=4), $7.5x10^{-7}$M (n=6), and $7.5x10^{-6}$M (n=6) PTH was normalized to the control. PTH produced a maximal increase of 99.2% ± 24% at $7.5x10^{-6}$M. (*) The % increase of the L-Ca^{2+} current is significantly different than at the concentrations of $2.5x10^{-9}$M and $7.5x10^{-9}$M PTH. ($p<0.05$ - Newman-Keul's).

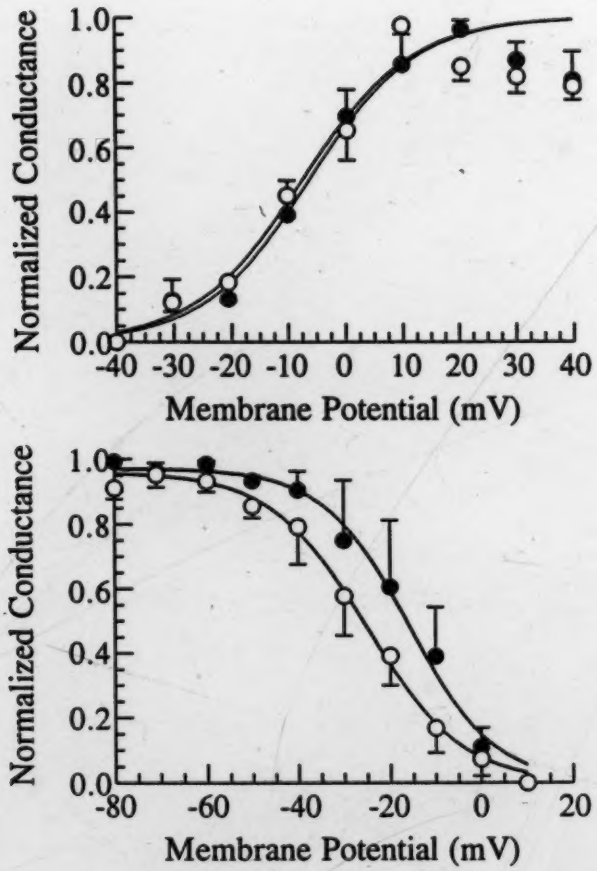

Fig. III-9 A. Steady-state activation (n=5) as described by the normalized conductance of the L-type Ca^{2+} channel current in UMR 106 cells before (closed circles) and after (open circles) the addition of 6.25×10^{-8}M PTHrP. The magnitudes of the normalized conductance were determined from tail currents. The steady-state activation of the L-type Ca^{2+} channel currents were not significantly changed by PTHrP when fitted with a Boltzmann function.

B. Steady-state inactivation (n=4) as described by the normalized conductance of the L-type Ca^{2+} channel current in UMR 106 cells before (closed circles) and after (open circles) the addition of 6.25×10^{-8}M PTHrP. The magnitudes of the normalized conductance were determined from tail currents. The steady-state inactivation of the L-type Ca^{2+} channel currents were not significantly changed by PTHrP when fitted with a Boltzmann function.

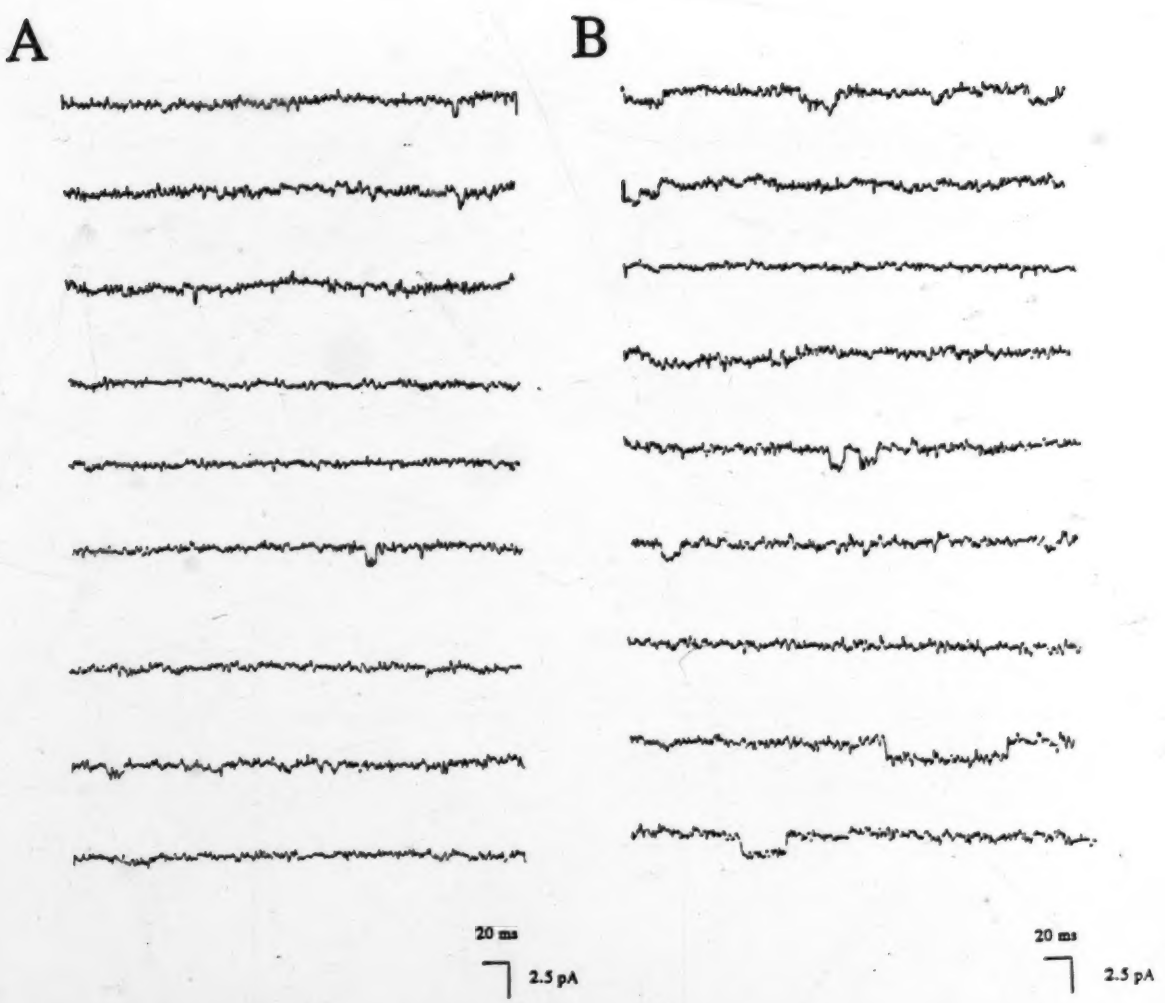

Fig. III-10 A. Single L-type Ca^{2+} channel recording from a UMR 106 cell. The traces were obtained from a cell-attached patch. A downward deflection represents an open state of the channel. The membrane patch was held at -50 mV and depolarized to +10 mV. The amplitude of the L-type Ca^{2+} channel current was approximately -0.85 pA. To promote the opening of the channel, $1x10^{-6}$M Bay K 8644 was included in the pipette solution. Currents were recorded 5-10 min. after the patch was obtained. Only the first 9 of the 16 sweeps are shown.

B. Single L-type Ca^{2+} channel recording from a UMR 106 cell after the addition of $1x10^{-9}$M PTHrP. The traces were obtained from the same cell as in Fig. III-9A. Since the effect of PTHrP was usually seen at approximately 15 min., the currents were recorded 15 min. after the addition of PTHrP. The amplitude of the L-type Ca^{2+} channel current remained at approximately -0.85 pA. The pipette solution contained $1x10^{-6}$M Bay K 8644 to promote the channel opening. After the addition of PTHrP, it appears that the number of channel openings and the channel open time increased. Only the first 9 of the 16 sweeps are shown. In A and B, the currents were filtered at 300 Hz.

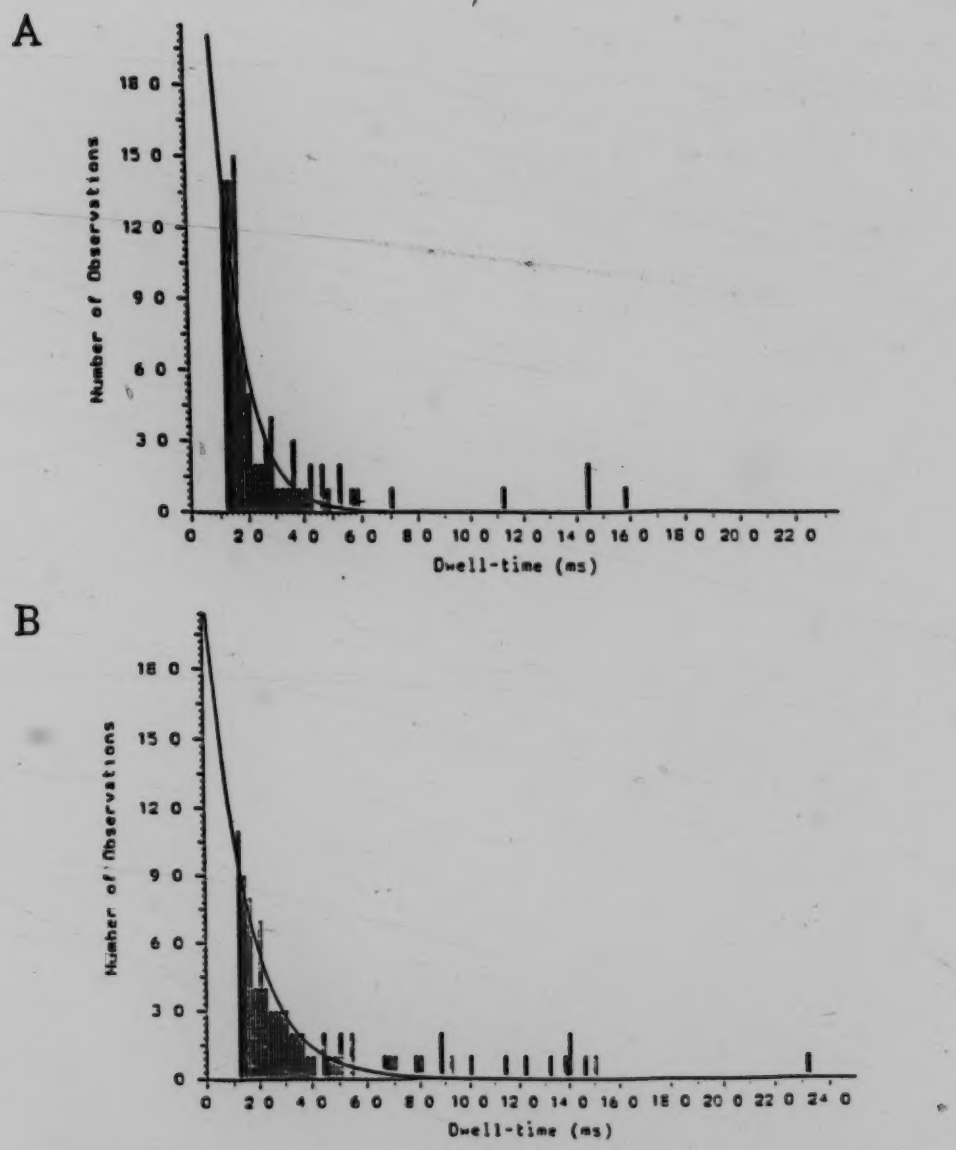

Fig. III-11 **A.** Open time distribution of the single L-type Ca^{2+} channel in a UMR 106 cell. The distribution was obtained from patches that were held at -50 mV and depolarized to +10 mV (see Fig. III-10A). The distribution was obtained from 32 separate 250 ms depolarizations. Most openings are brief and $\tau = 0.92$ ms.

B. Open time distribution of the single L-type Ca^{2+} channel after the addition of $1x10^{-9}$M PTHrP in a UMR 106 cell. The distribution was obtained from patches that were held at -50 mV and depolarized to +10 mV (see Fig. III-10B). The distribution was obtained from 32 separate 250 ms depolarizations. After the addition of PTHrP, the open times were longer as $\tau = 1.56$ ms.

Chapter IV

Results - Part II:

Second messenger mechanisms by which PTHrP modulates L-type calcium channels in UMR 106 cells

4.1 Introduction

It is known that voltage-dependent Ca^{2+} channels can be modulated by intracellular signalling systems that involve protein kinase phosphorylation of the channel proteins and subsequent altering of their gating (ie. open or closed state) (Curtis *et al.*, 1985; Braña *et al.*, 1993). Once a ligand binds to its receptor on the plasma membrane of a target cell, it may stimulate a cascade of events, such as the activation of a second messenger pathway(s), and subsequent activation of protein kinases. These protein kinases phosphorylate ion channels, such as Ca^{2+} channels, in the plasma membrane, and thereby modulate their state. For example, in smooth muscle cells of a rat tail artery, it was found that PTH activated the cAMP/PKA pathway, and PKA subsequently inhibited the L-type Ca^{2+} channels (Wang *et al.*, 1993). In addition, Chang *et al.* (1991) discovered that a PKC activator, 4β-phorbol 12-myristate 13-acetate (PMA) increased the L-type Ca^{2+} channel current in neonatal rat cardiac myocytes.

In Chapter III, both PTHrP and PTH were shown to significantly increase the L-type Ca^{2+} channel current in UMR 106 cells. Since PTHrP increased the L-type current, it was of interest to investigate the second messenger pathway(s) which are activated by

PTHrP. This chapter focuses on the investigation of second messenger pathways through which PTHrP modulates L-type Ca^{2+} channels in UMR 106 cells. The two effects on the L-type Ca^{2+} channel current were used as a comparison of the potency of PTHrP and PTH on the L-channel currents. In this chapter, experiments were undertaken to examine the roles of PKA, PKC, and PKG (another pathway linked to a G-protein involving the activation of guanylate cyclase, cGMP, and subsequently PKG) in PTHrP regulation of L-type channels.

4.2 Experimental Design

The whole cell version of the patch clamp technique and [cAMP]$_i$ measurement were used in these experiments as described in Chapter II. To determine the role of second messengers in the effect of PTHrP on the L-type Ca^{2+} channel current, activators and inhibitors of PKA, PKC, and PKG were used in the following experiments.

4.3 Results

4.3.1. Effect of 8-bromo-cAMP on the L-type calcium channel current in UMR 106 cells

To begin to assess the possible involvement of the adenylate cyclase/PKA pathway in PTHrP modulation of L-type Ca^{2+} channels in UMR 106 cells, 8-bromo-cAMP, a PKA activator, was applied to the extracellular solution. 8-bromo-cAMP is a membrane permeable cAMP analogue. In this experiment, the membrane was held at -40 mV and the peak inward current was monitored. Once the maximum current developed (ie. dialysis

was complete), an I-V relationship was obtained and 8-bromo-cAMP was added to the external solution. When the L-type current reached a maximum after the addition of one concentration of 8-bromo-cAMP, an I-V was obtained again. This protocol was used until 5 concentrations of 8-bromo-cAMP, $5x10^{-5}$M, $1x10^{-4}$M, $2.5x10^{-4}$M, $5x10^{-4}$M, and $1x10^{-3}$M were applied to the same cell. As shown in Fig. IV-1, 8-bromo-cAMP decreased the L-type Ca^{2+} channel current and the decrease was concentration-dependent, with a maximal decrease in the current occurring at $1x10^{-3}$M. At this concentration, 8-bromo-cAMP decreased the current to 65.6±8.7% (n=4) compared to the control level. The inhibition of the current was not due to run-down of the cell, as Fig. III-5 illustrates no significant run-down in cells after 25 min. Since PTHrP and PTH increase the L-type Ca^{2+} channel current in UMR 106 cells, this experiment suggests that the increase in current is not cAMP-dependent. In addition, these findings indicate that 8-bromo-cAMP negatively modulates L-type Ca^{2+} currents in UMR 106 cells.

4.3.2. The extracellular application of Rp-cAMPs on the effect of PTHrP on the L-type calcium channel current in UMR 106 cells

Rp-cAMPs, a specific inhibitor of PKA, was used to examine the effects of an inhibition of PKA on the modulation of L-type Ca^{2+} channel currents by PTHrP. In this experiment, the membrane was held at -40 mV and the peak current was monitored. Once the current reached its maximum, an I-V relationship was obtained and $1x10^{-9}$M PTHrP was added to the external solution. Another I-V relationship was measured after the maximal effect of PTHrP occurred (approximately 15 min.). At this point, $1x10^{-4}$ M Rp-

cAMPs was added to the extracellular solution. To allow time for Rp-cAMPs to diffuse from the external solution to the cell, the Ca^{2+} current was recorded for a period of 23 to 25 min in order to observe the effects of Rp-cAMPs within this time period. After this time, if no effect was seen, it was assumed that the drug did not affect PTHrP modulation of L-type Ca^{2+} channels. Rp-cAMPs was applied externally since the cells did not consistently respond to PTHrP; however, if Rp-cAMPs was included in the pipette solution and there was no increase in the current in the presence of PTHrP, it would not be known whether the inhibition was due to the presence of Rp-cAMPs, or whether the cell was not responding to PTHrP. Fig.IV-2 illustrates the effect of externally-applied Rp-cAMPs on a PTHrP-induced increase in the L-type current. Rp-cAMPs had no effect on the L-type current after a PTHrP-induced increase in the current (n=2). This suggests that the cAMP/PKA pathway does not play a major role in positively or negatively modulating the PTHrP-induced increase in the L-type Ca^{2+} current at this concentration of PTHrP (1 x 10^{-9}M).

4.3.3. Effect of forskolin on the L-type calcium channel current in UMR 106 cells

As a further means to investigate the role of the adenylate cyclase/PKA pathway in PTHrP modulation of L-type channels, forskolin, an adenylate cyclase activator, was added to the extracellular solution. In this experiment, the membrane was held at -40 mV and the peak inward Ca^{2+} current was monitored. After the current reached a maximum, an I-V relationship was obtained and 1x10^{-6}M forskolin was added to the extracellular

solution. Fig.IV-3 illustrates an inhibition by forskolin of the L-type Ca^{2+} current by 20.8±4.7% (n=4) in UMR 106 cells. Again, this provides evidence that PTHrP-induced positive modulation of L-type Ca^{2+} channels is not dependent upon the adenylate cyclase/PKA pathway, since forskolin inhibited the current and PTHrP enhanced the current. This is in agreement with the results in which 8-bromo-cAMP decreased the Ca^{2+} current, as it demonstrates that the cAMP/PKA pathway is not involved in the PTHrP-induced increase in the L-type Ca^{2+} current.

4.3.4. Effect of PTHrP and PTH on cAMP accumulation in UMR 106 cells

It is well documented that PTHrP and PTH increase cAMP accumulation in many cell lines, including UMR 106 cells. In order to confirm that this physiological event was still occurring in these cells, and that the previous results seen were not due to a disfunctional group of cells, the effect of 5 concentrations of PTHrP and PTH on $[cAMP]_i$ accumulation was examined. A radioimmunoassay was used to measure $[cAMP]_i$ (see Materials and Methods). Fig.IV-4 illustrates that a 10 min separate treatment of $1x10^5$ cells with $1x10^{-7}$M, $1x10^{-8}$M, $1x10^{-9}$, $1x10^{-10}$M, and $1x10^{-11}$M PTHrP and PTH caused a concentration-dependent increase in cAMP, with a maximal increase seen at $1x10^{-8}$M PTHrP and PTH (n=5). PTHrP and PTH had equal potencies in increasing cAMP in UMR 106 cells. In a repeated experiment, similar results were obtained (data not shown).

Thus, PTHrP and PTH can increase cAMP accumulation in UMR 106 cells, but that the positive modulation of L-type Ca^{2+} channels by these hormones is not through the cAMP pathway. This demonstrates the complexity of PTHrP and PTH-induced bone

resorption.

4.3.5. Effect of PTHrP and PTH, in the presence of IBMX on intracellular cAMP accumulation in UMR 106 cells

It is of interest to determine how PTHrP and PTH cause an increase in [cAMP]: whether it is due to an increase in production of cAMP or whether it is due to an inhibition of the enzyme that breaks down cAMP (ie. phosphodiesterase). Therefore, IBMX, a phosphodiesterase inhibitor, was added to the cells in the presence of PTHrP and PTH to determine if there was an additional increase in cAMP accumulation compared to the control with IBMX alone. In this experiment, a radioimmunoassay was used to determine $[cAMP]_i$ in UMR 106 cells. Fig. IV-5 demonstrates that $1x10^{-3}$M IBMX, in the absence of PTHrP and PTH, increased cAMP accumulation by approximately 20,000-fold. In the presence of IBMX, $1x10^{-9}$M, PTHrP and PTH further potentiated the increase in cAMP accumulation. Since PTHrP and PTH caused a further increase in cAMP accumulation in the presence of IBMX, this suggests that PTHrP and PTH increase cAMP accumulation by increasing the production of cAMP, rather than inhibiting phosphodiesterase activity.

4.3.6. Effect of 4β-phorbol 12-myristate 13-acetate (PMA) on the L-type calcium channel current in UMR 106 cells

Since the adenylate cyclase/PKA pathway does not appear to be involved in positive PTHrP modulation of L-type channels, an alternative biochemical pathway was examined. In this case, PMA, a PKC activator, was added to the extracellular solution to observe its

effects and the possible role that the PLC/PKC pathway has on modulating the L-type Ca^{2+} channels in UMR 106 cells. In this experiment, the membrane was held at -40 mV and the peak current was monitored. Once the maximum current occurred, an I-V relationship was obtained and PMA was added to the external solution. This protocol was used successively until 3 concentrations of PMA, $1x10^{-8}$M, $1x10^{-7}$M, and $1x10^{-6}$M, were applied to the same cell. Fig.IV-6 demonstrates a concentration-dependent decrease in the current. This suggests that PTHrP-induced increases in the L-type Ca^{2+} channel current in UMR 106 cells is not through PKC activation. Since PMA activates only PKC of the PLC pathway, this does not eliminate the possibility that PTHrP channel modulation is mediated by another portion of the PLC pathway, such as IP_3.

4.3.7. The interaction between PMA and PTHrP on the L-type calcium channel current in UMR 106 cells

To determine a direct role of PKC in modulating the PTHrP-induced increase in the L-type Ca^{2+} current, PMA was added to the external solution after the effect of PTHrP was observed. In this experiment, the membrane was held at -40 mV and the peak inward current was monitored. After the current reached its steady-state maximum, an I-V relationship was obtained and the cell was treated with $1x10^{-9}$M PTHrP. When the effect of PTHrP on the L-type current reached a maximum steady state, $1x10^{-6}$M PMA was added. The results in Fig. IV-8 show that in a single cell, after PTHrP increased the current 65% from the control, the addition of PMA decreased the current 21% from the control, or 86% from the current amplitude after the effect of PTHrP. Similar results were

obtained from 3 other cells (data not shown). These results suggest that PKC negatively modulates the PTHrP-induced increase in L-type Ca^{2+} channel currents in UMR 106 cells.

4.3.8. The effect of db-cGMP on the L-type calcium channel current in UMR 106 cells

To investigate the possibility that PTHrP and PTH modulate L-type Ca^{2+} channels through the guanylate cyclase/PKG pathway, the effects of cGMP on the L-type channels in UMR 106 cells was examined. In this experiment, the membrane was held at -40 mV and the peak inward current was monitored. Once the current reached a steady-state maximum, an I-V relationship was obtained and db-cGMP was added to the external solution. This protocol was used to apply a second concentration of cGMP to the same cell. Fig. IV-9 demonstrates that the L-type Ca^{2+} channel current was decreased in the presence of two concentrations of db-cGMP, $1x10^{-5}$M, and $1x10^{-4}$M. At $1x10^{-5}$M db-cGMP, the current was decreased by $43\pm7\%$ and at $1x10^{-4}$M db-cGMP, the current was decreased by $38\pm2.5\%$. This suggests that PTHrP-induced increases in the L-type channel current in UMR 106 cells is not through the guanylate cyclase/PKG pathway, since PTHrP increases the current and cGMP decreases the current.

4.4 Discussion

This chapter focused on investigating the intracellular mechanism(s) by which PTHrP modulates L-type Ca^{2+} channel currents in UMR 106 cells. One experiment involved the addition of 8-bromo-cAMP to determine if it could mimic the effect of PTHrP

in increasing the L-type Ca^{2+} channel current. Instead, this PKA agonist decreased the L-type current in a concentration dependent manner. This suggests that the positive modulation of L-type channels by PTHrP in UMR 106 cells is not through the adenylate cyclase/PKA pathway, since PTHrP had an opposite effect than 8-bromo-cAMP on the current. It would also suggest that 8-bromo-cAMP negatively modulates L-type Ca^{2+} channels in UMR 106 cells.

In addition, to determine the role of PKA in modulating the PTHrP-induced increase in the L-type current, Rp-cAMPs, a PKA inhibitor, was added to the external solution. In this experiment, Rp-cAMPs did not enhance or inhibit the current after a PTHrP-induced increase in the current. This suggests that cAMP/PKA does not play a major role in the modulation of L-type Ca^{2+} channels. However, it would be expected that Rp-cAMPs would increase the current since 8-bromo-cAMP decreased the current. One possible explanation for there being no rise in the current was that Rp-cAMPs may not have completely inhibited PKA.

To support a negative modulatory effect of the cAMP pathway PTHrP regulation of L-type Ca^{2+} channels in UMR 106 cells, forskolin, an adenylate cyclase activator, was added to the extracellular solution. It was found that forskolin inhibited the current, once again, indicating that PTHrP-mediated increase in the L-type Ca^{2+} channel current is not through the adenylate cyclase/PKA pathway.

A further experiment involved the determination of cAMP accumulation upon stimulation of UMR 106 cells with five concentrations of PTHrP and PTH. Since an increase in cAMP has been a well-documented phenomenon, this experiment was

performed to confirm that the phenotype of cells, with regards to the cAMP response, had not changed. It was observed that PTHrP and PTH increased $[cAMP]_i$ in a concentration-dependent manner. In addition, PTHrP and PTH equipotently increased cAMP accumulation. Since these hormones were equipotent in stimulating $[cAMP]_i$, but not in modulating the L-type Ca^{2+} current (ie. PTHrP was more potent in increasing the current), this further supports the suggestion that the positive modulation of L-type Ca^{2+} channels is not through cAMP and that the cellular response generated by these hormones could be a combination of an increase in cAMP and an increase in $[Ca^{2+}]_i$ through plasma membrane Ca^{2+} channels. Although the Ca^{2+} channels appear to not be modulated by the cAMP pathway, this does not mean that the cAMP signalling system does not play a role in other aspects of PTHrP and PTH hormone signalling in UMR 106 cells.

Another experiment was performed to determine whether PTHrP and PTH increase $[cAMP]_i$ by increasing cAMP production, or by inhibiting the breakdown of cAMP by phosphodiesterases. To do this, the effect of IBMX, a phosphodiesterase inhibitor and thereby a cAMP activator, on cAMP accumulation in the presence of PTHrP and PTH was determined. It was found that IBMX increased $[cAMP]_i$ by 20,000 times. In the presence of IBMX, PTHrP and PTH further potentiated the increase cAMP accumulation by approximately four fold. This suggests that PTHrP and PTH increase cAMP by increasing the production of cAMP rather than inhibiting its breakdown by phosphodiesterases.

Since the adenylate cyclase pathway appears to not be involved in positive L-type Ca^{2+} channel modulation by PTHrP, another pathway had to be investigated. Therefore, the effect of PMA, a PKC activator, on the L-type Ca^{2+} channel current in UMR 106 cells

was examined. It was found that PMA inhibited the current in a concentration-dependent manner, suggesting that PTHrP does not increase the L-type channel current through PKC. However, since PMA only activates PKC, and bypasses other steps along the PLC pathway, such as the activation of phosphoinositides, the role that other portions of the PLC pathway play in PTHrP and PTH modulation of the L-type channels remains unknown.

A further experiment looked at the interaction between PTHrP and PMA on the L-type Ca^{2+} channel current in UMR 106 cells. It was discovered that after PTHrP increased the L-type current, PMA inhibited the current to an amplitude below the control level. This suggests that PKC decreases the PTHrP-induced increase in the current, possibly by phosphorylating the Ca^{2+} channels and causing a decrease in their open states.

Finally, the involvement of another signalling system, the guanylate cyclase/cGMP pathway, in PTHrP modulation of L-type Ca^{2+} channels was investigated. The results indicated that PTHrP does not positively modulate L-type Ca^{2+} channels in UMR 106 cells through this pathway, since cGMP inhibited the L-type current and PTHrP increased the current.

The mechanism of PTHrP and PTH to increase the L-type Ca^{2+} current in UMR 106 cells is complex. In this study, the activation of PKA, PKC and PKG all demonstrated an inhibition of the current, which excludes them from direct positive modulation of the L-type current. The possibility of other pathways that may be involved include:

1. A G-protein that is directly linked to an L-Ca^{2+} channel to cause an increase in the current.

2. A different component of the PLC pathway, such as phosphoinositides, that may not only stimulate a release of Ca^{2+} from intracellular stores, but may also open plasma membrane Ca^{2+} channels. In addition, a release of intracellular stores may cause an opening of Ca^{2+} channels in order to replenish the $[Ca^{2+}]_i$.

3. An unknown signal transduction pathway.

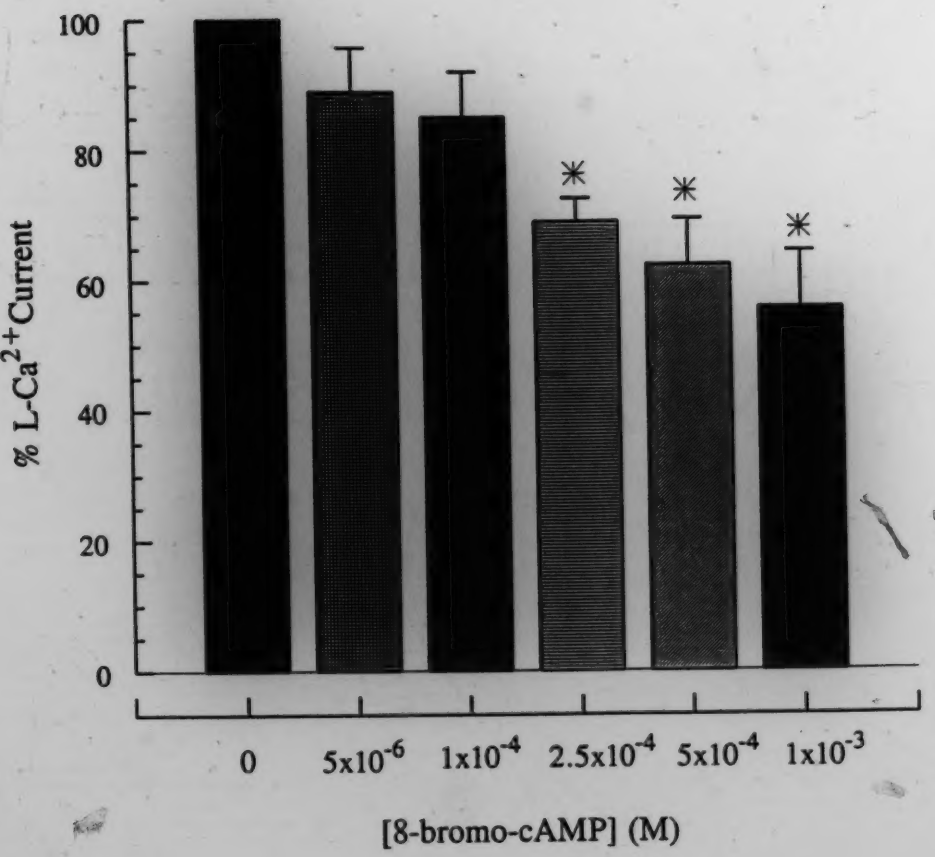

Fig. IV-1. The effect of 5 concentrations of 8-bromo-cAMP on the L-type Ca^{2+} channel current in UMR 106 cells. The membrane was held at -40 mV and the peak inward current was measured before and after the cumulative addition of $5x10^{-6}$M, $1x10^{-4}$M, $2.5x10^{-4}$M, $5x10^{-4}$M, and $1x10^{-3}$M 8-bromo-cAMP and the effect of the drug on the current was measured as a % of the control (100%) ($\bar{x} \pm$ S.E.M., n=4). In this experiment, 8-bromo-cAMP decreased the L-type Ca^{2+} current in a concentration-dependent manner. At a concentration of $1x10^{-3}$M, 8-bromo-cAMP maximally decreased the L-type Ca^{2+} channel current by 44.4±8.7%. (*) The % increase in the L-Ca^{2+} current is significantly different than at the concentrations of 0M, $5x10^{-6}$M and $1x10^{-4}$M 8-bromo-cAMP. ($p<0.05$ - Newman-Keul's).

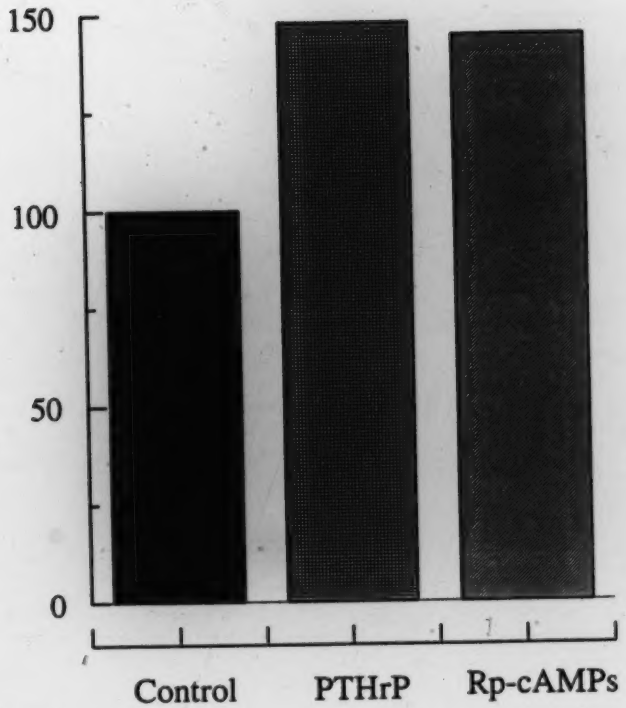

Fig. IV-2. The effect of externally applied Rp-cAMPs, a PKA inhibitor, on the PTHrP-induced increase of the L-type Ca^{2+} channel current in UMR 106 cells. The membrane was held at -40 mV and the peak inward current was measured before and after the addition of $1x10^{-9}$M PTHrP. Once the current amplitude reached a maximum from the effect of PTHrP (15 min.), $1x10^{-4}$M Rp-cAMPs was applied. Rp-cAMPs was allowed 23-25 min. to diffuse into the cell and to have an effect before it was assumed that it had no effect on the current. Any change in current was compared as a percentage of the control (100%). In this experiment, PTHrP increased the L-current by 48% (n=2). In the presence of PTHrP, Rp-cAMPs had no effect on the current.

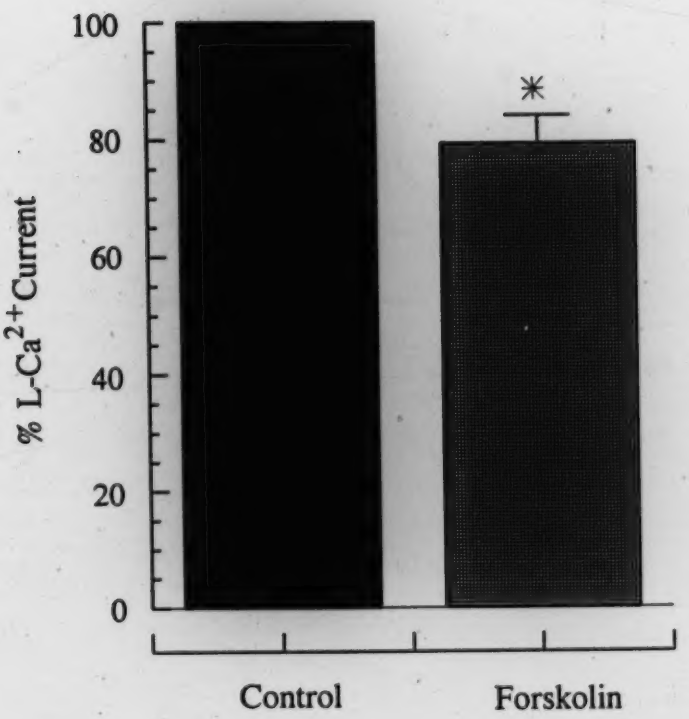

Fig. IV-3. The effect of forskolin, an adenylate cyclase agonist, on L-type Ca^{2+} channel currents in UMR 106 cells. The membrane was held at -40 mV and the peak inward current was measured before and after the addition of $1x10^{-6}$M forskolin. The effect of forskolin on the current was measured as a % of the control current (100%). In this experiment, forskolin decreased the L-channel current by 20.8±4.7% ($\bar{x}$ ± S.E.M., n=4). (* $p<0.05$).

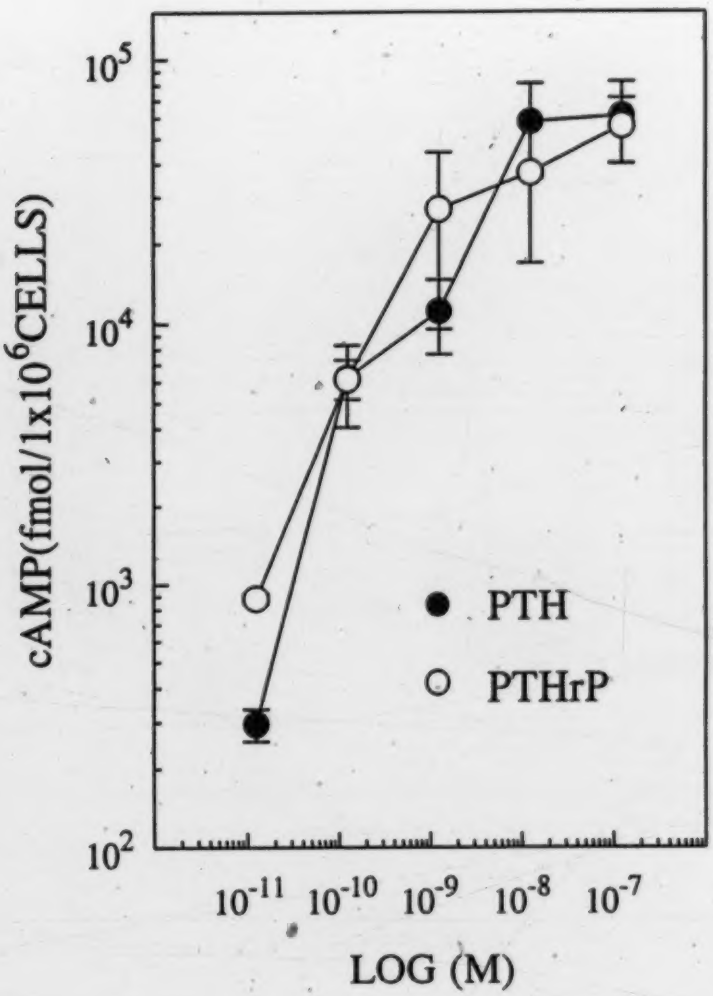

Fig. IV-4. The effect of PTHrP and PTH on cAMP accumulation in UMR 106 cells. 5 concentrations of PTHrP and PTH were used: $1x10^{-7}$M, $1x10^{-8}$M, $1x10^{-9}$M, $1x10^{-10}$M and $1x10^{-11}$M (n=5). PTHrP and PTH equipotently increased intracellular cAMP as a function of concentration.

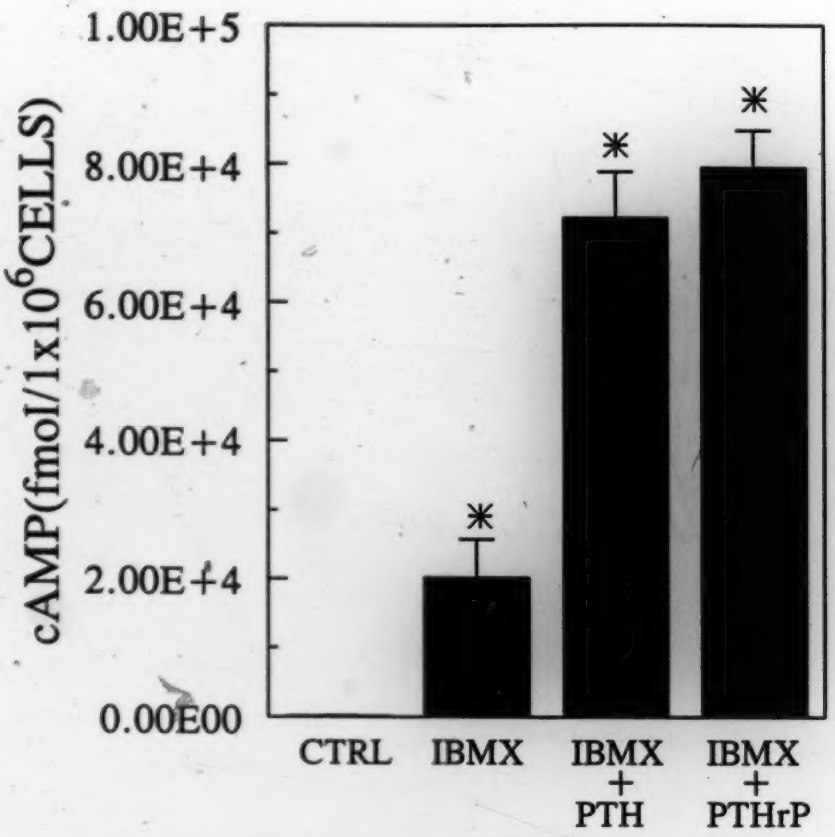

Fig. IV-5. The effect of PTHrP and PTH, in the presence of IBMX, a phosphodiesterase inhibitor, on intracellular cAMP concentrations in UMR 106 cells. $1x10^{-3}$M IBMX, by itself, increased cAMP significantly. $1x10^{-9}$M PTHrP and PTH, in the presence of $1x10^{-3}$M IBMX, further potentiated the increase in cAMP. (*$p<0.05$).

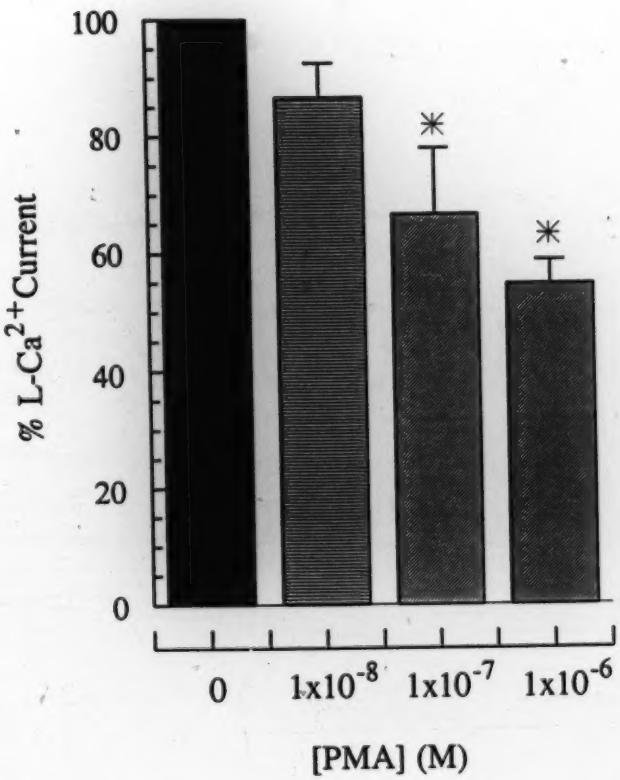

Fig. IV-6. The effect of PMA, a PKC agonist, on the L-type Ca^{2+} channel current in UMR 106 cells. The membrane was held at -40 mV and the peak current amplitude was measured before and after the cumulative addition of $1x10^{-8}$M, $1x10^{-7}$M, and $1x10^{-6}$M PMA ($\bar{x} \pm$ S.E.M., n=4). The effect of PMA on the current was compared as a % of the control current (100%). In this case, PMA decreased the L-type current as a function of concentration, with a maximal decrease of 45.4 ± 4.1% ($\bar{x} \pm$ S.E.M., n=4) at a concentration of $1x10^{-6}$M. (*) The % increase of the L-Ca^{2+} current is significantly different than at the concentrations of 0M and $1x10^{-8}$M PMA. ($p<0.05$ - Newman-Keul's).

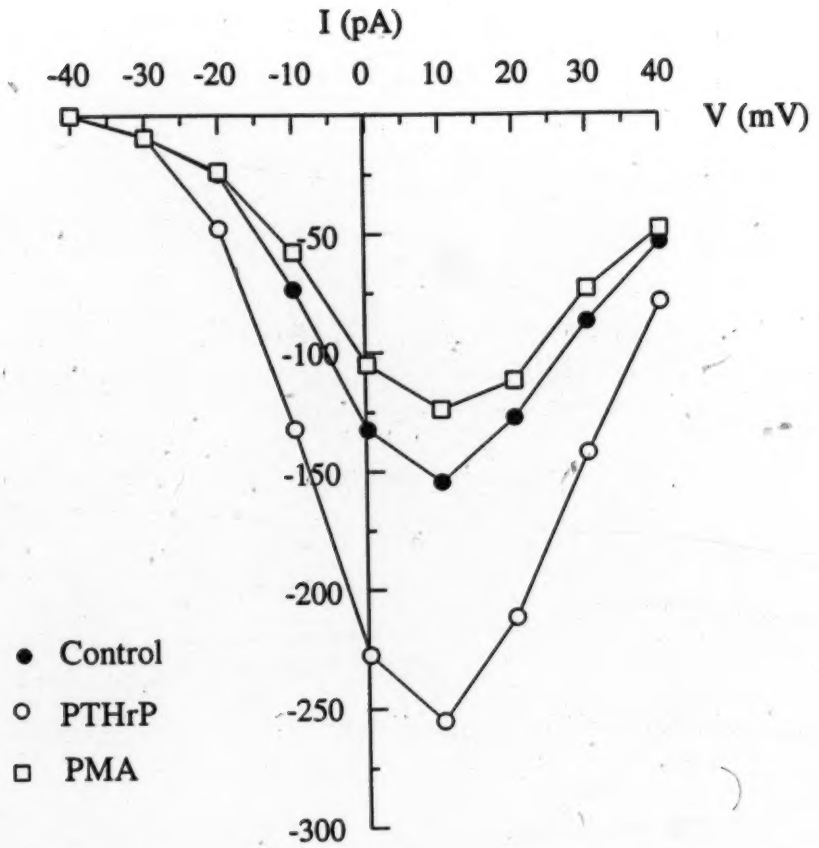

Fig. IV-7. The effect of PMA, in the presence of PTHrP, on the L-type Ca^{2+} channel current in a UMR 106 cell. The membrane was held at -40 mV and the peak inward current was measured before (closed circles) and after (open circles) the addition of $1x10^{-9}$M PTHrP. PTHrP increased the current amplitude from -154 pA to -255 pA. After the addition of $1x10^{-6}$M PMA, the current decreased from -255 pA to -123 pA.

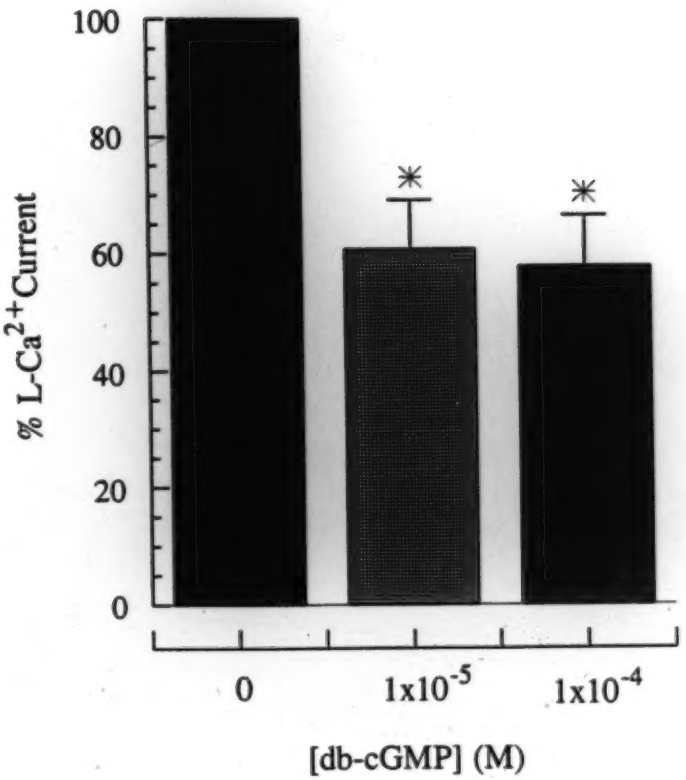

Fig. IV-8. The effect of db-cGMP on the L-type Ca^{2+} channel current in UMR 106 cells. The membrane was held at -40 mV and the peak current was measured before and after the cumulative addition of $1x10^{-5}$M and $1x10^{-4}$M db-cGMP. The effect of db-cGMP on the current was measured as a % of the control current (100%) ($\bar{x}$ ± S.E.M., n=4). In this experiment, db-cGMP decreased the L-channel current. There was no significant difference between the two concentrations in inhibiting the current, with a nearly maximal decrease of 40±8.4% occurring at a concentration of $1x10^{-5}$M db-cGMP. (*) The % increase of the L-Ca^{2+} current is significantly different than the control. ($p<0.05$ - Newman-Keul's).

Chapter V

General discussion and summary

5.1. General discussion

5.1.1. Effects of PTHrP and PTH on L-type calcium channel currents in UMR 106 cells

PTHrP and PTH both stimulate bone resorption (Moseley *et al.*, 1995). The stimulation of bone resorption appears to be extremely complex, with multiple biochemical pathways contributing to its activation. It is thought that changes in $[Ca^{2+}]_i$ concentration is an important factor in stimulating the bone resorption process, however, the source of Ca^{2+} remains controversial (ie. an influx of Ca^{2+} through plasma membrane Ca^{2+} channels and/or a release of Ca^{2+} IP_3-sensitive endoplasmic reticulum stores). Voltage-dependent Ca^{2+} channels play an important role in Ca^{2+} entry in many cells, including cardiac myocytes (Rampe *et al.*, 1991), smooth muscle cells (Wang *et al.*, 1991), and bone cells (Chesnoy-Marchais *et al.*, 1988; Karpinski *et al.*, 1989). Karpinski *et al.* (1989) found that L-type Ca^{2+} channels were the predominant form of Ca^{2+} channels in UMR 106 cells, an osteoblastic osteosarcoma cell line. In most cells, these channels are regulated by second messenger pathways; therefore, it is important to determine how PTHrP and PTH modulate these channels. In order for voltage-dependent Ca^{2+} channels to be activated, the membrane must be depolarized. It has been shown that PTH depolarizes osteoblast membranes (Ferrier *et al.*, 1988; Chesnoy-Marchais *et al.*, 1989) and it is likely that this

occurs through receptor-operated channels or second messenger-operated channels. Therefore, once the L-type Ca^{2+} channels are activated, PTHrP and PTH, through second messenger pathways yet to be determined, modulate activity of these channels.

One study (Fritsch *et al.*, 1994) looked at the modulation of L-type Ca^{2+} currents in newborn rat calvaria by PTH (1-34); however, at this point the modulation of L-type Ca^{2+} channel currents by PTHrP and PTH in UMR 106 cells, an osteoblast-like cell line that represents a mature form of bone, has not yet been investigated. In addition, the effect of PTHrP on L-type Ca^{2+} channel currents has not been studied until the present.

The present study, using UMR 106 cells, characterized the effects of PTHrP on L-type Ca^{2+} channel currents and uses the effects of PTH on the current as a means of comparison. This thesis demonstrated that in UMR 106 cells, PTH significantly increased the L-type Ca^{2+} channel current and that the increase was concentration-dependent, in support of the findings of Fritsch *et al.* (1994). PTHrP was also found to increase the L-type current in a concentration-dependent manner. It is known that PTHrP and PTH bind to the same "classical" PTHrP/PTH receptor on osteoblast cells and stimulate bone resorption. Thus, it could be suggested that since PTHrP and PTH both increase $[Ca^{2+}]_i$ concentrations, the mechanism of these hormones to enhance the bone resorptive process may be through the regulation of L-type Ca^{2+} channels in the plasma membrane of bone cells.

Several studies have reported widely varying potencies between PTHrP and PTH in stimulating bone resorption. Although an equal increase in $[cAMP]_i$ stimulated by PTHrP and PTH has been reported (Blind *et al.*, 1993), the study in this thesis

demonstrated that PTHrP was more potent than PTH in increasing the L-type Ca^{2+} current. Since both an increase in $[cAMP]_i$ and an increase in $[Ca^{2+}]_i$ (via plasma membrane Ca^{2+} channels or release of $[Ca^{2+}]_i$ stores) are thought to be involved in bone resorption, and the two hormones are not equally potent in the modulation of $[Ca^{2+}]_i$, but are equally potent in increasing $[cAMP]_i$ this would suggest three possibilities: 1) PTHrP and PTH activate different signal transduction pathways in osteoblasts. Although one common pathway for the two hormones is the cAMP pathway, the increase in $[Ca^{2+}]_i$ (which may be modulated by either the cAMP pathway and/or the PLC pathway or some other signal transduction mechanism), is not equally stimulated by PTHrP and PTH. 2) PTHrP and PTH may have different affinities for the PTHrP/PTH receptor, suggesting that PTHrP may bind with a higher affinity than PTH. At present, the available literature has only compared the bone-resorbing potencies of the two hormones, but not their binding affinities for receptors within the same cell line. However, some studies have demonstrated that different fragments of the PTHrP and PTH peptides have varying affinities for the PTHrP/PTH receptor from tissue to tissue (Demay *et al.*, 1985; Juppner *et al.*, 1988). 3) The possibility of other receptors has not been eliminated. Although other receptors for PTHrP and PTH on osteoblast membranes have not been found, it is possible that other receptors for these hormones exist. For instance, recently, Orloff *et al.* (1995) discovered the existence of a second PTHrP/PTH receptor in normal keratinocytes and squamous carcinoma cell lines. This receptor is activated by N-terminal fragments of PTHrP and PTH, leading to increases in $[Ca^{2+}]_i$, but not cAMP. Therefore, if other receptors for PTHrP and PTH exist on osteoblast membranes, the activation of different pathways for the two hormones is a

definite possibility.

When ligands bind to receptors and modulate Ca^{2+} channels in membranes, they often change the gating of the channel. PTHrP may modify the gating of the channel by increasing the open time of the channel and/or by increasing the frequency of channel opening, thereby allowing a greater influx of Ca^{2+} than normal. Another possibility is that PTHrP may increase the number of Ca^{2+} channel openings and/or the probability of channel opening. In this thesis, it was found that PTHrP increased both the number of channel openings and the open time of the channel.

Although PTHrP and PTH usually increased the L-type Ca^{2+} channel current in UMR 106 cells, these hormones sometimes had no effect on the current. Fritsch *et al.* (1994) also found an inconsistency in the ability of PTH to induce an increase in the Ca^{2+} current. In approximately 30% of the cases, PTH increased the current; however, in many cases, it had no effect or even decreased the current. Reasons for this inconsistent effect of PTHrP and PTH has been attributed to the osteoblasts being in different stages of the cell cycle. For instance, Bizzarri *et al.* (1994) reported that in UMR 106 cells, the ability of PTH to increase $[Ca^{2+}]_i$ depended on the stage of the cell within its replicative cycle. In S phase, which is the stage in which DNA replication is occurring but cell division has not begun, cells were more responsive to PTH than cells in the G1 phase, the stage in which mRNA is being produced but cell division has not begun. In addition, they found that the S phase PTH response was mostly dependent on Ca^{2+} influx through the membrane, whereas PTH-induced Ca^{2+} transients by cells in the G1 phase did not depend upon Ca^{2+} influx through membrane channels. These observations could be interpreted to

represent a model of PTH signal transduction which predicts two different signalling modes, the S mode and the G1 mode. These differences in the cell cycle in UMR 106 cells makes it more difficult to determine the mechanism of signal transduction by PTHrP and PTH. In addition to an inconsistency in the ability of PTHrP and PTH to increase Ca^{2+} due to the cell cycle, the UMR 106 cell phenotype has been shown to be heterogeneous (Guenther *et al.*, 1989). It has been shown that after UMR 106 cells have been cultured for a period of time, their phenotype changes. This adds an additional complication to studying PTHrP and PTH modulation of Ca^{2+} channels, since there may be phenotypically different cell subtypes within the population. Civitelli *et al.* (1992) suggested that the mechanisms by which $[Ca^{2+}]_i$ responses are generated may be due to the competence of each single cell to bind PTHrP and PTH may be due to the nonuniform distribution of functional receptors over the cytoplasmic surface as the cells progress into later passages. The studies on cell cycle and osteoblastic phenotype demonstrate that the PTHrP and PTH-activation of the pathway which stimulates an influx of Ca^{2+} is cell-cycle dependent, possibly by cell-cycle related sensitivity of the pathway. Although the transformed UMR 106 cell line probably reproduces some of the events occurring during bone development, future studies using normal, phenotypically defined cell models would be necessary to complement the present studies in order to fully understand the basis of PTHrP and PTH-induced bone resorption.

5.1.2. The investigation of second messenger mechanisms by which PTHrP modulates L-type calcium channel currents in UMR 106 cells

The modulation of L-type Ca^{2+} channels by second messengers has been studied in several types of cells, including heart (Rampe *et al.*, 1989), pituitary (Rampe *et al.*, 1989), vascular smooth muscle (Wang *et al.*, 1991), and neonatal rat calvaria (Fritsch *et al.*, 1994). It has been shown that L-type Ca^{2+} channels are regulated by the phosphorylation of channel proteins (Tsien *et al.*, 1986). The phosphorylation of channel proteins is due to the activities of protein kinases, such as protein kinase A and protein kinase C (Curtis *et al.*, 1985; Hell *et al.*, 1993). This change in the activity of the channel, due to phosphorylation, can cause an increase or decrease in the current, depending on the cell type (Chang *et al.*, 1991). If both kinases are activated after ligand binding, the integration of the effects of PKA and PKC on the channel will determine the final activity of the channel.

The second messenger pathways by which PTHrP and PTH modulate L-type Ca^{2+} channel currents in UMR 106 cells remain unclear and controversial. While PTHrP and PTH have been shown to consistently increase $[cAMP]_i$ (Blind *et al.*, 1993; Civitelli *et al.*, 1994), and this pathway was originally thought to stimulate bone resorption, PTHrP and PTH-induced increases in $[Ca]_i$ have also been implicated in the bone-resorptive process (Yamaguchi *et al.*, 1987 a,b); however, the PTHrP and PTH-induced increase in $[Ca^{2+}]_i$ is inconsistent (Bizzarri *et al.*, 1994; Boland *et al.*, 1986; Wiltink *et al.*, 1993; Civitelli *et al.*, 1992; Ljunggren *et al.*, 1992).

In this study, the second messenger system by which PTHrP modulates the L-type

Ca^{2+} current was investigated. Since a classical effect of PTHrP and PTH on target cells is to increase $[cAMP]_i$, we studied the effects of 8-bromo-cAMP, a cAMP analogue, and forskolin, an adenylate cyclase activator, on the L-type current, reasoning that their effects would mimic those of PTHrP. It was found that 8-bromo-cAMP decreased the L-type current, suggesting that PTHrP-mediated increases in Ca^{2+} influx are not through the cAMP/PKA pathway. In addition, forskolin decreased the L-type current, again indicating that PTHrP-induced increases in the Ca^{2+} current are not due to cAMP/PKA activation. cAMP is, therefore, only one of the many modulators in the complex bone-resorbing process.

To further investigate the inhibitory role of the cAMP pathway in regulating L-type Ca^{2+} channels after PTHrP stimulation, Rp-cAMPs, a PKA inhibitor, was added to determine if the L-type Ca^{2+} current would be increased. After a PTHrP-induced increase in the current, it was found that Rp-cAMPs had no effect on the current, suggesting that the cAMP/PKA pathway does not play a significant role in the modulation of the PTHrP or PTH-induced increase in the L-type Ca^{2+} current at this PTHrP concentration (1×10^{-9}M). Since it would be expected that Rp-cAMPs would increase the L-type current if 8-bromo-cAMP decreased the L-type current, a possible explanation for this result may be that Rp-cAMPs did not completely inhibit PKA. Other studies have also confirmed that PTH does not increase $[Ca^{2+}]_i$ through its actions to stimulate cAMP (Fraser *et al.*, 1988; Scoble *et al.*, 1985; Khalifa *et al.*, 1983; Hruska *et al.*, 1986; Goligorsky *et al.*, 1986). Again, this does not mean that the PTHrP and PTH-induced rise in $[cAMP]_i$, that is consistently seen, is not involved in bone resorption. In fact, in the presence of Rp-

cAMPs, bone resorption in calvarial bones from newborn mice, is inhibited in a concentration-dependent manner (Ljunggren *et al.*, 1993; Sugimoto *et al.*, 1994). This would suggest that the dominant pathway in bone resorption is the cAMP/PKA system and that the PLC/PKC pathway plays a contributory role by increasing $[Ca^{2+}]_i$. However, the same study would have to be performed on a more mature osteoblast model before any major conclusions could be drawn.

It is well established that a classical effect of PTHrP and PTH is to increase $[cAMP]_i$ in osteoblasts; therefore, to confirm that the cells were responding properly, and that the phenotype had not changed, the cells were stimulated with PTHrP and PTH and $[cAMP]_i$ was measured. It was found that PTHrP and PTH both stimulated an increase in $[cAMP]_i$, indicating that the cell phenotype likely had not changed. In addition, this study demonstrated an equal potency between PTHrP and PTH in stimulating an increase in $[cAMP]_i$. These results confirm the study by Blind *et al.* (1993), who also found equal potencies for PTHrP and PTH in increasing $[cAMP]_i$. Also, these results suggest that since PTHrP and PTH increase $[cAMP]_i$ and patch-clamp studies have demonstrated an 8-bromo-cAMP-induced decrease in the L-type Ca^{2+} current, cAMP likely negatively modulates L-type Ca^{2+} channels in UMR 106 cells. This study also found that the PTHrP-induced increase in cAMP accumulation is likely due to an increase in the production of cAMP and not due to an inhibition of phosphodiesterase activity.

Since the cAMP/PKA pathway appears to be a negative modulator, and therefore is not the major contributor in PTHrP positive modulation of L-type Ca^{2+} channels in the membrane of UMR 106 cells, other pathways, such as the PLC/PKC pathway, were

investigated. PKC has been shown to modulate ion channel activity in many cell types. In fact, in UMR 106 cells, Yamaguchi *et al.* (1987b) discovered a protein kinase C-activated Ca^{2+} channel which could also be activated by PTH, suggesting that the modulation of Ca^{2+} channels by PTH is through PKC. However, in the present study, it was found that PMA, a PKC activator, inhibited the L-type current, which suggests that PKC is not involved in the positive modulation of L-type channels in the membrane. Instead, it may play a role in the down-regulation or desensitization (via phosphorylation) of the L-type channels in UMR 106 cells. This finding was confirmed by Li *et al.* (1996) in which PMA inhibited the L-type Ca^{2+} channel current in UMR 106 cells. In this study, in the presence of PTHrP, PMA still inhibited the L-type Ca^{2+} channel current, suggesting that PKC is not involved in the PTHrP-stimulated increase in the L-type current, and when administered in *in vitro* situations, decreases the PTHrP-induced increase in the L-type current. Fura-2 studies performed by Yamaguchi *et al.* (1987a,b), however, do not support these findings, since stimulation of PKC with the phorbol ester, TPA, mimicked the PTH-induced rise in $[Ca^{2+}]_i$, which depended on the presence of extracellular Ca^{2+} and was inhibited by Ca^{2+} channel blockers in UMR 106 cells. Further studies must be performed to further elucidate the role of PKC in PTHrP and PTH modulation of L-type Ca^{2+} channel currents in these cells.

Although our results suggest little or no role for PKC in PTHrP and PTH positive modulation of L-type channels, other studies have suggested a role for the PLC pathway in the stimulation of bone resorption by these hormones. For instance, Babich *et al.* (1989) concluded that UMR 106 membranes possess a G protein-sensitive phosphoinositide-

specific phospholipase C and that this signal transduction pathway contributes to the skeletal actions of PTH. In addition, Tatrai *et al.* (1994) found that inhibition of PLC attenuated the Ca^{2+} transients induced by PTH, and that the inhibition occurred in the presence of extracellular Ca^{2+}. They concluded that the PLC pathway plays a role in Ca^{2+} transients elicited by PTH, and that it may be involved in the opening of L-type membrane Ca^{2+} channels. The involvement of inositol phosphates in the modulation of L-type Ca^{2+} channels should be an area of future investigation.

In addition, although PKC may not be involved in PTHrP modulation of L-type channels in UMR 106 cells, it could play some other role in the bone resorptive process. For instance, Iida-Klein *et al.* (1989) found that UMR 106 cells possessed a classic, phorbol ester-activated PKC system which was highly Ca^{2+} and phospholipid dependent. It was shown in the present study that PKC, when activated with phorbol esters, concentration-dependently inhibits the PTHrP increase in the L-type Ca^{2+} channel current. Hence, it could be suggested that if PTHrP acts through the PLC pathway, PKC negatively modulates the L-type Ca^{2+} current in a similar manner to cAMP/PKA. Perhaps PKC plays a different role in PTHrP and PTH stimulation of bone resorption, possibly by up-regulating the cAMP response, as one study suggested (Kovacs *et al.*, 1995) and preliminary results in this study have demonstrated (data not shown).

Ca^{2+} current modulation by cGMP have been described in a number of cell types. For instance, the Ca^{2+} current in snail neurons is enhanced by cGMP-dependent protein kinase (cGMP) (Fritsch *et al.*, 1994), however, further studies have failed to demonstrate Ca^{2+} channel regulation by cGMP. The present study demonstrated that cGMP decreased

the L-type current, suggesting an inhibitory modulation of L-type Ca^{2+} channels by cGMP in UMR 106 cells. This experiment confirmed a study by Fritsch *et al.* (1994) in which the involvement of cGMP in PTH modulation of L-type channels was tested in newborn rat calvaria and similar results were obtained.

In this study, the ability of PTHrP to increase the L-type Ca^{2+} channel current cannot be ascribed to the cAMP/PKA pathway, to PKC, or to PKG. The transient hypocalcemia that occurs prior to the better known hypercalcemic effects of PTHrP and PTH may be an important factor in rationalizing why cAMP is not involved in the increase in $[Ca^{2+}]_i$. This hypocalcemic effect induced by PTHrP and PTH is due to the action of these hormones to induce cellular uptake of Ca^{2+} (Boland *et al.*, 1986; Schofl *et al.*, 1991). The fact that the Ca^{2+} response does not appear to be mediated by cAMP is supported by two lines of evidence: 1) cAMP does not mimic the actions of PTH to increase $[Ca^{2+}]_i$ in renal tubular cells (Hruska *et al.*, 1986). 2) forskolin, an adenylate cyclase activator, did not mimic the actions of PTH to elevate $[Ca^{2+}]_i$ in UMR 106 cells in this study and other studies (Reid *et al.*, 1987; Short *et al.*, 1991). The observation that PTHrP increases $[cAMP]_i$ and $[Ca^{2+}]_i$ suggests that it stimulates both the cAMP pathway and the PLC pathway. Since cAMP appears to not be involved in the positive modulation of L-type Ca^{2+} channels in the plasma membrane, this implies that the PLC pathway may be involved in the modulation of L-type Ca^{2+} channels. Since PKC regulation of these channels was also shown to decrease the PTHrP-stimulated increase in the L-type Ca^{2+} current in the present study and by Li *et al.* (1996), this suggests that some other aspect of the PLC pathway may be involved in the positive modulation of these channels. For instance, the pathway of

hydrolysis of phosphatidylinositides (PIs), which results in the generation of Ca^{2+}-mobilizing inositol phosphates may be a likely target of PTHrP and PTH action. In the past 20 years, the involvement of the PI system as a major pathway for many hormones has been elucidated (Michell, 1975; Berridge, 1984; Abdel-Latif, 1986). In UMR 106 cells (Civitelli *et al.*, 1988), ROS 17/2.8 (Cosman *et al.*, 1989), opossum kidney cells (Ruth *et al.*, 1986), renal tubular cells (Hruska *et al.*, 1987), and canine renal cortical tubular cells (Coleman *et al.*, 1990), it has been shown that PTH elevates inositol triphosphates. Several studies have demonstrated that Ca^{2+} influx can be triggered by Ins(1,3,4,5)P_4 and by Ins(1,4,5)P_3 or by the combined action of the two (Irvine *et al.*, 1986; Michell, 1986; Kuno *et al.*, 1987; Whitaker *et al.*, 1984). Therefore, a role of inositol phosphates in PTHrP and PTH modulation of L-type channels is a definite possibility.

In addition, there is evidence for Ca^{2+}-activated Ca^{2+} channels that open in direct response to an elevation in $[Ca^{2+}]_i$ (von-Tscharner *et al.*, 1986). The release of $[Ca^{2+}]_i$ stores may stimulate a Ca^{2+}-calmodulin mechanism which may phosphorylate membrane channels and change their state. However, this hypothesis is unlikely since $[Ca^{2+}]_i$ was chelated to a low concentration in the study presented in this thesis. There is also the possibility that inositol phosphates modulate L-type Ca^{2+} channel currents through an unknown mechanism. It has also been suggested that there may be several G proteins that are responsible for the coupling of PTHrP and PTH to PLC and subsequent phosphoinositide (PI) hydrolysis. For instance, Ca^{2+}-mobilizing hormones, including PTH, that utilize the PI pathway are enhanced by GTP or GTPγS, a Gprotein activator (Babich *et al.*, 1989; Coleman *et al.*, 1990; Cosman *et al.*, 1989).

Although PLC pathway activation and subsequent PI hydrolysis seems a likely explanation for PTHrP and PTH modulation of L-type Ca^{2+} channels, further studies would have to be performed to confirm this hypothesis. In addition to the inositol phosphate component of the PLC pathway, there are other remaining possibilities of PTHrP regulation of L-type Ca^{2+} channel currents:

1. PTHrP modulation of L-type Ca^{2+} channels may be due to a G protein that is directly coupled to a PTHrP/PTH receptor and Ca^{2+} channels in the plasma membrane. Although this seems possible, it is unlikely, since in the single channel analysis in this study, it was found that PTHrP caused longer openings and the openings were more frequent. This can be accounted for by diffusable second messengers. In addition, effects that are caused by direct G protein coupling usually occur more quickly.
2. The stimulation of an unknown signal transduction pathway.

PTHrP and PTH appear to activate at least two messenger pathways in osteoblasts (see Fig. V-1). It was suggested earlier in this thesis that since PTHrP and PTH were found to have different potencies on the L-type Ca^{2+} channel current, the two hormones may be operating through different second messenger pathways. This study did not investigate this hypothesis, as it only looked at the possible second messenger pathways for PTHrP modulation of L-type Ca^{2+} channels in UMR 106 cells. This would be an area of future study. This hypothesis is, however, supported by Short *et al.* (1990) in which retinoic acid, a constituent of serum, was necessary to induce $[Ca^{2+}]_i$ rises by PTH, but not by PTHrP. They suggested that because of this, the mechanisms of action of PTHrP and

PTH differ with respect to rises in $[Ca^{2+}]_i$. These findings are also supported by Yamada *et al.* (1989) who demonstrated that PTH mediated $[Ca^{2+}]_i$ changes, but PTHrP did not in a murine osteoblastic cell line, MCT3T3-E1.

The precise role that each of the cAMP/PKA and PLC pathways play in PTHrP and PTH-stimulated bone resorption remains to be elucidated. It could be that the pathways play roles in both bone formation and resorption, since PTH has been found to have both catabolic and anabolic functions. The next step will be to elucidate the pathway which positively modulates the PTHrP-induced increase in the L-type Ca^{2+} current. At this point, the cAMP/PKA, PKC, and cGMP/PKG pathways have been investigated and found to have negative modulatory roles in PTHrP and PTH-induced increases in L-type channel currents. However, since these hormones consistently increase $[cAMP]_i$, it is likely that the cAMP/PKA pathway plays some role in PTHrP and PTH-induced bone resorption. The possibility that other receptors will be identified in the osteoblast membrane still exists. Work over the next few years should elucidate further receptors and other signal transduction pathways that are involved in PTHrP and PTH-stimulated bone resorption.

5.1.3. Clinical Significance

At the present time, researchers are investigating the many roles of PTHrP in physiology. This peptide, however, is predominantly known for its role in humoral hypercalcemia of malignancy (HHM); therefore, by elucidating the signal transduction pathways through which this hormone stimulates bone resorption, inhibitors could be used to target specific second messengers in order to prevent its hypercalcemic effect. As

further studies uncover the cellular mechanisms of this hormone, a clinical significance for PTHrP will become more clear.

5.2. Summary

5.2.1. PTHrP and PTH were found to increase the L-type Ca^{2+} channel current in UMR 106 cells as a function of concentration.

5.2.2. PTHrP was found to be significantly more potent than PTH in increasing the L-type Ca^{2+} channel current in UMR 106 cells.

5.2.3. PTHrP had no significant effect on the steady-state activation and inactivation of the L-type Ca^{2+} channel. Single channel analysis demonstrated an increase in the time constant fitted to the open time distribution of the channel after the addition of PTHrP. P(o), a relative measure of the time that a channel spends in the open state, increased from 0.007 to 0.038 after the addition of PTHrP. This suggests that the PTHrP-induced increase in the L-type Ca^{2+} current is due to an increase in the open time of the L-type Ca^{2+} channels. In addition, from observation, it was seen that the number of channel openings increased after the addition of PTHrP. This suggests that an increase in the L-type Ca^{2+} current may also be due to an increase in the number of L-type Ca^{2+} channels that are opening.

5.2.4. 8-bromo-cAMP, a PKA agonist, was shown to decrease the L-type Ca^{2+} channel current in UMR 106 cells in a concentration-dependent manner. In addition, forskolin, an adenylate cyclase activator, decreased the L-type channel current. This suggests that the positive modulation of L-type Ca^{2+} channel currents by PTHrP is not by cAMP/PKA.

5.2.5. Rp-cAMPs, a PKA inhibitor, had no effect on the PTHrP-induced rise in the L-type Ca^{2+} current, suggesting that the cAMP pathway is not significantly involved in PTHrP modulation of these channels or that Rp-cAMPs did not completely inhibit PKA.

5.2.6. PMA, a PKC activator, decreased the L-type Ca^{2+} channel current as a function of concentration. In addition, after a PTHrP-induced rise in the L-type Ca^{2+} channel current, PMA decreased the current. This suggests that PKC negatively modulates the PTHrP-induced increase in the L-type Ca^{2+} current in UMR 106 cells.

5.2.7. db-cGMP, a cGMP analogue, inhibited the L-type Ca^{2+} channel current, suggesting that the guanylate cyclase pathway negatively modulates L-type Ca^{2+} channels in UMR 106 cells and it is not the pathway by which PTHrP increases the L-type Ca^{2+} current.

5.2.8. PTHrP and PTH were both found to equally stimulate $[cAMP]_i$

accumulation in a concentration-dependent manner. This indicated that the phenotype of the UMR 106 cells had not changed, since this response is a classical one of both hormones. This also demonstrated that the hormones had equal potency in stimulating $[cAMP]_i$ accumulation, unlike their potencies to increase the L-type Ca^{2+} current. In addition, it suggests that since 8-bromo cAMP decreased the L-type Ca^{2+} current, the cAMP/PKA pathway may provide negative feedback and negatively modulate L-type Ca^{2+} currents in UMR 106 cells.

5.2.9. PTHrP and PTH, in the presence of IBMX, a phosphodiesterase inhibitor, further increased cAMP accumulation, suggesting that the mechanism of these hormones to increase cAMP is to increase the production of cAMP rather than inhibit the enzyme that breaks down cAMP.

5.3. Significance

5.3.1. This is the first study to investigate the effect of PTHrP and PTH on L-type Ca^{2+} channel currents in UMR 106 cells. A demonstration of the effect of PTHrP on the single L-type Ca^{2+} current in UMR 106 cells was also performed for the first time in the present study. It is also the first study to investigate the modulation of L-type Ca^{2+} channel currents by PTHrP in UMR 106 cells, and to eliminate the possibilities of positive modulation through cAMP/PKA, PKC, or cGMP/PKG.

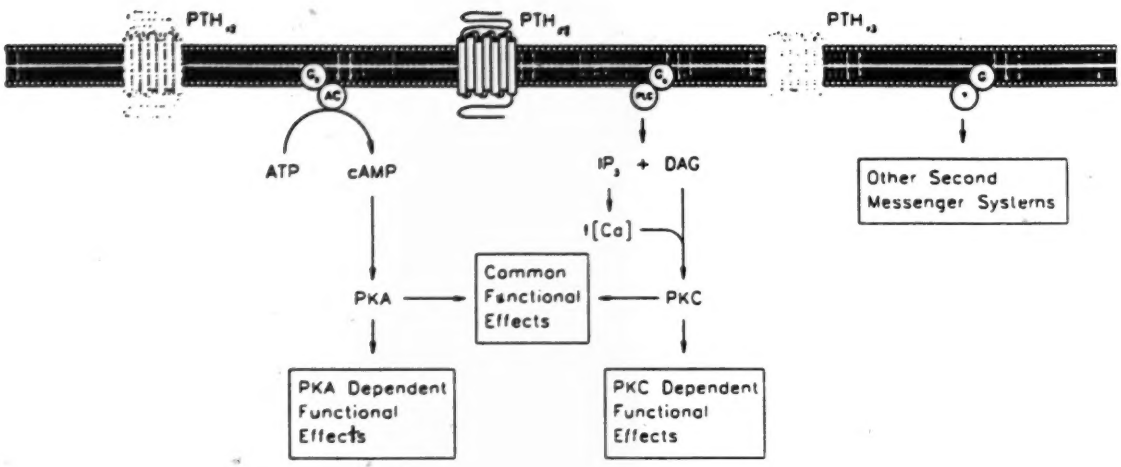

Fig. V-1. Schematic representation of PTH-mediated activation of second messenger systems. Cloned PTH receptors (PTH_{r1}) couple PTHrP and PTH to both adenylyl cyclase and phospholipase C in target cells. Additional receptors for PTH are likely to exist. These potential receptors (PTH_{r2} and PTH_{r3}) may couple more specifically to either second messenger system or may be present only in certain tissues (Coleman *et al.*, 1994).

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